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Review

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Pharmacogenomics

BRAF in metastatic colorectal cancer: the future starts now

BRAF mutations are detectable in about 5–15% of metastatic colorectal cancer (mCRC) patients and represent a clear negative prognostic factor. While in BRAF-mutated (BRAFmt) metastatic melanoma TKI target therapies (BRAF and MEK inhibitor), both alone or in combination, have shown significant efficacy, in BRAFmt CRC single-agent BRAF-inhibitors as well as chemotherapy seem to be ineffective. The critical role of EGFR in CRC and its multiple downstreaming pathways seem to be involved in this lack of response. In recent years, preclinical investigations and retrospective studies slowly increased our knowledge on BRAFmt CRC. This review analyses preclinical data and discusses several clinical trials in order to explore new therapeutic strategies targeting BRAFmt mCRC.

Keywords: BRAF • colorectal cancer • immunotherapy • target therapy

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BRAF function

RAF is a family of serine/threonine kinases [1]. It consists of three isoforms, ARAF, BRAF and CRAF, and plays a central role in intracellular signaling and cell growth, being a downstream effector of the GTPases KRAS within the MAPK signaling pathway [2]. The MAPK pathway is a group of kinases made up of RAS, RAF, MAPK/MEK1/2 and ERK [3]. Activated ERK binds to transcription factors, leading to regulation of gene expression, promoting growth, differentiation, survival and proliferation. RAF proteins act as signals relays from activated RAS proteins via MEK1/2 to ERK1/2, the key effectors of the pathway [4]. Under normal conditions, activation of the MAPK pathway occurs through ligand binding to tyrosine kinase receptors such as EGFR, leading to dimerization and autophosphorylation [5] (Figure 1). Following receptor dimerization, adaptor proteins undergo phosphorylation that leads to the activation of RAS GTPases. GTP-bound RAS recruits and activates the RAF proteins, which phosphorylate and thus activate MEK1 and MEK2 which in turn phosphorylate and activate ERK1 and ERK2 resulting in activation of transcription factors and thereby in cell proliferation, survival and differentiation [6]. The constitutive activation of the MAPK pathway, through deregulation of the RAS–RAF–MEK–ERK signaling cascade, has been demonstrated to play an important role for tumor development, being one of the most common events in malignancies. Activating BRAF mutations have been described to allow constitutive activation of the MAPK pathway, leading to hyperactive proliferative signaling [7].

Prognostic role of BRAF mutations in metastatic colorectal cancer

BRAF somatic mutations were first reported in 2002 [8]. Since then a number of different mutations have been described in many malignancies. The presence of BRAF mutations in mCRC is quite uncommon, being detected in about 4.7–15.2% of all tumors [9,10]. BRAF mutations occur in two regions of the BRAF kinase domain, the glycine-rich loop and the activations segment. Although several BRAF mutations were evaluated in many studies [11–13], the biological effects of each mutations were not

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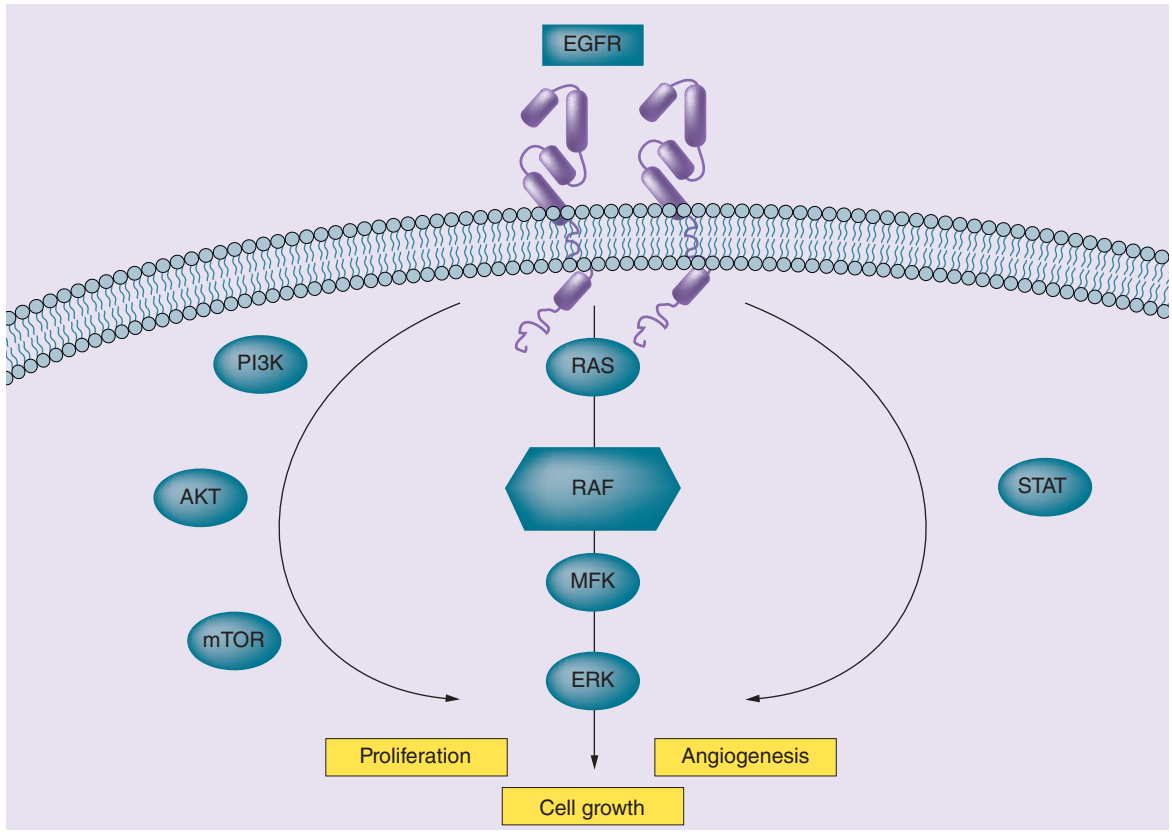

Figure 1. EGFR/MAPK signaling pathway in colorectal cancer cell.

fully understood. Interesting to note for example that the substitution of alanine for T598 or D593 reduced or abolished kinase and transforming activities [8,11,13]. The most common mutation is the missense mutation V600E, a T-A change at nucleotide 1799 (codon 600, exon 15), which encodes an amino acid change, the substitution of valine for glutamic acid, within the activation segment [14]. The oncogenic V600E mutation introduces a negative charge that mimics the phosphorylation events that occur at threonine 598 and serine 601, overcoming the requirement for phosphorylation at these sites in activation of BRAF [1]. This amino acid change within the activation segment disrupts the autoinhibitory mechanism and converts BRAF into its active form, allowing the constitutive activation of the MAPK signaling pathway [14]. The BRAF V600E mutation, which accounts for ~80% of BRAF mutations, and the second most common mutation V600K account for most of all BRAF mutations in colorectal cancer (CRC). BRAF mutations are frequently observed in CRC with microsatellite instability (MSI). Approximately 50% of the microsatellite unstable tumors are BRAF mutated whereas few microsatellite stable tumors harbor BRAF mutations [15]. In mCRC BRAF V600E mutation has been associated with a worse outcome [10]. The prognostic role of BRAF in mCRC patients was prospectively investigated in a study including 524 patients with known BRAF mutational status [16]. BRAFmt tumors were 11% and demonstrated a significantly poorer median survival (10.4 vs 34.7 months, $p < 0.001$). BRAFV600E mutation analysis was also carried out in 504 mCRC patients treated with systemic therapies in everyday clinical practice [17]. A significant reduction in progression-free survival (PFS; 4.1 vs 11.6 months; $p < 0.001$) and overall survival (OS; 14.0 vs 34.6 months; $p < 0.001$) was found in the BRAFmt group. In the multivariate analysis, BRAFV600E mutation strongly correlated with an inferior PFS (HR: 4.1, 95% CI: 2.7–6.2; $p < 0.001$) and OS (HR: 5.9, 95% CI: 3.7–9.5; $p < 0.001$). Also the results from the MRC FOCUS trial confirmed a significant reduction in OS for BRAFmt mCRC ($p = 0.0002$) [18].

BRAF-mutated metastatic colorectal cancer: current treatment

Anti-EGFR therapy: is there a role?

In the last years the introduction of anti-EGFR therapy determined an important focus on EGFR cellular pathway in CRC. Many studies underlined the negative predictive role of RAS mutations for anti-EGFR therapy [19,20]. In fact, mutated RAS status is predic-

tive of resistance to anti-EGFR monoclonal antibodies (anti-EGFR MoAbs) cetuximab and panitumumab and mutation testing for RAS is performed, in mCRC, to select patients with wild-type tumors for treatment with anti-EGFR MoAbs. Despite the low incidence, an increasing number of studies documented a negative prognostic impact and a negative predictive role of BRAF mutation in mCRC, which therefore identifies a group of patients with a worse outcome [10]. Up to now, no prospective randomized clinical trial has ever evaluated the relationship between BRAF mutation status and resistance to anti-EGFR MoAb in mCRC patients. Instead the predictive role of BRAF mutation for anti-EGFR treatment has been investigated with retrospective subset analysis of prospective trials. During last years, several studies compared anti-EGFR MoAb-based regimens and chemotherapy alone in treatment of mCRC patients. Multiple retrospective subgroup analyses have been performed to systematically review the benefit of anti-EGFR MoAb according to BRAF mutational status, suggesting lack of antitumor activity in presence of BRAF mutations. However, the conclusions were controversial, besides, these studies contained a relatively small number of BRAFmt patients. In order to improve the statistical power, those analyses have been summarized in seven meta-analyses which investigate the predictor role of BRAF in anti-EGFR therapy (Tables 1 & 2). The first meta-analysis was published in 2011 by Mao *et al.* It included 11 retrospective studies with a total of 922 patients treated with anti-EGFR MoAb-based therapy or monotherapy. This meta-analysis demonstrated a larger response benefit of anti-EGFR MoAbs in BRAFwt over BRAFmt patients, though did not compare other indicators such as PFS and OS [21]. In 2012 Bokemayer *et al.* presented a pooled analysis of individual patient data from both CRYSTAL and OPUS trials, investigating the association between tumor KRAS and BRAF mutation status and survival benefit of adding anti-EGFR MoAb cetuximab to standard first-line regimen. The meta-analysis showed a survival benefit of anti-EGFR MoAbs for BRAFwt (HR for PFS 0.64, 95% CI: 0.52–0.79; $p < 0.0001$; HR for OS 0.84, 95% CI: 0.71–1; $p = 0.048$) but not for BRAFmt on a selected KRASwt population [22]. A subsequent study performed a direct comparison, including 21 trials with 4616 patients, on effects of anti-EGFR MoAbs, mainly in second- rather than in first-line, between patients with BRAFmt and BRAFwt mCRC. Patients with BRAFwt showed improved PFS and OS compared with BRAFmt both in unselected (HR for PFS 0.38, 95% CI: 0.29–0.51; HR for OS 0.35, 95% CI: 0.29–0.42) and KRASwt population (HR for PFS 0.29, 95% CI: 0.19–0.43; HR for OS 0.26, 95% CI: 0.2–0.35). A response benefit for BRAFwt, with an improved overall response rate (ORR), was observed in KRASwt (RR: 0.31, 95% CI: 0.18–0.53), but not in unselected patients [23]. A further meta-analysis of four randomized clinical trials (CRISTAL, OPUS, NORDIC VII and AIO KRK-0104) evaluated the relationship between BRAF V600E mutation and ORR of anti-EGFR MoAbs for first-line treatment in mCRC patients. In BRAFmt carriers adding anti-EGFR MoAb to chemotherapy was similar to chemotherapy alone both in KRASwt and unselected patients. Whereas in BRAFwt adding anti-EGFR MoAb to chemotherapy produced a clear benefit in ORR and this advantage was restricted to KRASwt patients (RR 1.48, 95% CI: 1.28–1.71) [24]. Moreover, a meta-analysis of 22 studies including 2395 patients affected by KRASwt (exon 2) mCRC treated with anti-EGFR MoAbs investigated the predictive role of BRAFmt in KRASwt tumors, showing a significantly lower ORR (RR: 0.29, 95% CI: 0.16–0.54) and shorter PFS (HR: 2.95, 95% CI: 1.89–4.61) and OS (HR: 2.52, 95% CI: 1.39–4.56) in BRAFmt versus BRAFwt patients [25]. In 2014 Wang *et al.* reviewed 7 studies and 1352 patients and showed a relation between BRAF V600E mutation and lack of response (RR 0.27, 95% CI: 0.10–0.70) and worse survival (HR for PFS 2.78, 95% CI: 1.62–4.76; HR for OS 2.54, 95% CI: 1.93–3.32) in KRASwt mCRC patients treated with anti-EGFR MoAbs [26]. Finally Pietrantonio *et al.* presented a meta-analysis examining the impact of MoAbs on ORR and survival in patients with RASwt/BRAFmt mCRC in order to evaluate the predictive role of BRAFmt in such population. Ten trials were included for a total of 463 RASwt/BRAFmt mCRC patients being analyzed. Overall, the addition anti-EGFR MoAb to chemotherapy in the BRAFmt subgroup did not significantly improved PFS, OS or ORR compared with control regimens [27]. In conclusion, the first six meta-analyses underlined the significantly advantage in ORR and survival of anti-EGFR therapy in KRASwt/BRAFwt patients when compared to KRAS/BRAFmt ones. Instead the Pietrantonio *et al.* meta-analysis points out, for the first time, how the addition of anti-EGFR therapy to cytotoxic therapy in KRASwt/BRAFmt patients does not improve response rate and survival. Overall these findings support the role of BRAFmt as predictor of resistance to anti-EGFR MoAbs and thus the need for BRAF mutational status assessment before initiation of treatment with anti-EGFR monoclonal antibodies.

Cytotoxic & antiangiogenetic therapy: is this the standard?

Despite huge advances in knowledge of molecular biology, to date, no effective therapeutic strategy is available in BRAFmt mCRC patients. However, dif-

Table 1. Meta-analysis comparing the anti-EGFR monoclonal antibody-based therapy effect in BRAF wild-type and BRAF-mutated colorectal cancer.

Study (year)	Included studies (n)	Patients analyzed (n)	Line of treatment with anti-EGFR	RR (95% CI; BRAF mt vs wt)		PFS HR (95% CI)		OS HR (95% CI)		Ref.
				RAS wt	RAS unselected	RAS wt (BRAF wt vs mt)	RAS wt (BRAF mt vs wt)	RAS wt (BRAF wt vs mt)	RAS wt (BRAF mt vs wt)	
Mao *et al.* (2011)	11	922	1, 2 or more	0.14 (0.04–0.53)	0.86 (0.57–1.30)	–	–	–	–	[21]
Yuan *et al.* (2013)	21	4616	1, 2 or more	0.31 (0.18–0.53)	–	0.29 (0.19–0.43)	–	0.26 (0.2–0.35)	–	[23]
Cui *et al.* (2014)	4	1245	1	0.43 (0.16–0.75)	0.45 (0.18–1.09)	–	–	–	–	[24]
Therkildsen *et al.* (2014)	22	2395	1, 2 or more	0.29 (0.16–0.54)	–	–	2.95 (1.89–4.61)	–	2.52 (1.39–4.56)	[25]
Wang *et al.* (2014)	7	1352	1, 2 or more	0.27 (0.10–0.70)	–	–	2.78 (1.62–4.76)	–	2.54 (1.93–3.32)	[26]

mt: Mutated; OS: Overall survival; PFS: Progression-free survival; RR: Relative risk; wt: Wild-type.

ferent evidences suggest that the adverse prognostic role of BRAF might be overcome if patients are treated aggressively. In a retrospective exploratory analysis of a Phase II trial, Masi *et al.* showed an interesting benefit of the combination of FOLFOXIRI (5-FU/LV + oxaliplatin + irinotecan) plus bevacizumab. This *post hoc* analysis, in a subgroup of 10 BRAFmt patients, reported remarkable findings for ORR (90%), median PFS (12.8 months) and median OS (23.8 months) with no significant differences compared with the BRAFwt subset [28]. These preliminary results suggested that an intensive upfront treatment might be a promising strategy to face the aggressiveness of BRAFmt mCRC. Drawing from these evidences, Loupakis *et al.* designed a prospective trial, with the aim to validate those retrospective data and to better estimate the potential impact of FOLFOXIRI plus bevacizumab as initial treatment for BRAFmt mCRC patients. In 15 BRAFmt patients a median PFS and OS of 9.2 and 24.1 months respectively was reported with an overall disease control rate (DCR) of 80% [29]. The impact of this intensive regimen has been also evaluated in the Phase III TRIBE trial. According to an exploratory subgroup analysis, the benefit provided by the addition of oxaliplatin to first-line FOLFIRI plus bevacizumab was independent of BRAF mutational status and a clinically relevant HR of 0.55 in favor of the triplet plus bevacizumab was reported in the subgroup of 28 BRAFmt patients [30]. Additionally, in this subgroup median PFS and OS was 7.5 and 19.1 months respectively with FOLFOXIRI plus bevacizumab and 5.5 and 10.8 months with FOLFIRI (5-FU/LV + irinotecan) plus bevacizumab [31]. Nevertheless, the actual contribution of the antiangiogenic therapy in this poor-prognosis subgroup is not clear, since the benefit provided by the addition of bevacizumab to FOLFOXIRI has never been investigated, whereas an important role might be attributed to oxaliplatin, which appeared more effective both *in vitro* and *in vivo* in CRC with mutated and hyperactive EGFR pathway [32,33]. Anyway, the identification of an effective treatment option for BRAFmt mCRC represents a major clinical need. Based on these data, an intensive first-line treatment might be considered a promising strategy to contrast the aggressiveness of this subtype of mCRC.

BRAF targeted therapy: preclinical studies

BRAF inhibitors, such as vemurafenib and dabrafenib, have produced response rates of ~50–80% in melanoma harboring BRAF V600E mutation, leading to US FDA approval and revolutionizing the treatment of this disease [34,35]. However, in CRC harboring BRAF V600E mutation, BRAF inhibitor monotherapy has proven to be ineffective, with response rates of

~5% [36]. Such striking disparity in sensitivity between these malignancies represents a critical challenge to develop effective therapies for BRAFmt CRC. Therefore, the reason for the different efficacy of vemurafenib between BRAFmt CRC and melanoma was extensively investigated in preclinical studies. Several data suggest that BRAF inhibitor insensitivity in BRAFmt CRC is driven by feedback reactivation of MAPK signaling following BRAF inhibitor treatment [37,38]. In BRAFmt CRC, the feedback reactivation of MAPK signaling appears to be driven by EGFR-mediated activation of RAS and CRAF [39]. Melanoma cells, deriving from the neural crest, express low levels of EGFR (expressed primarily in epithelial cancers) and are therefore not subject to this feedback activation. In order to explore the different sensitivity to BRAF inhibition between BRAFmt CRC and BRAFmt melanoma, Corcoran *et al.* evaluated the effects of vemurafenib on CRC and melanoma cell lines harboring BRAF V600 mutations [37]. BRAFmt CRC cell lines demonstrated decreased sensitivity to vemurafenib *in vitro*, mirroring the results from clinical trials. As opposed to what happens in BRAFmt melanoma cell lines, although treatment with vemurafenib slowed the growth of BRAFmt CRC cells compared with untreated controls, it failed to decrease viable cell number compared with pretreatment staring cell number in BRAFmt CRC cell lines. In fact vemurafenib treatment led to a transient phospho-ERK (P-ERK) suppression in CRC cell lines instead of the sustained suppression of P-ERK observed in melanoma cell lines. This finding is consistent with an EGFR-mediated reactivation of MAPK signaling in BRAFmt CRC. According to other evidences, this resistance mechanism seems to involve activation of RAS via EGFR, leading to greater levels of activated RAS and P-CRAF in BRAFmt CRC rather than in BRAFmt melanoma [40,41]. Inhibition of EGFR abrogated RAS activation, P-CRAF induction and P-ERK reactivation upon treatment with vemurafenib in BRAFmt CRC cells, suggesting that vemurafenib might produce sustained inhibition of mutant BRAF activity and suppression of ERK phosphorylation in the absence of EGFR-mediated feedback signals. Moreover, in order to investigate the molecular mechanism responsible for the intrinsic resistance of BRAFmt CRC to vemurafenib, Prahallad *et al.* performed an RNA-interference-based genetic screen in human cells searching for kinases whose knockdown synergizes with BRAFV600E inhibition [39]. Inhibition of EGFR through monoclonal antibody (cetuximab) or TKI (gefitinib or erlotinib) appeared strongly synergistic with BRAF inhibition in multiple BRAF V600E-mutant CRC, both *in vitro* and *in vivo*. Particularly, in cell lines, treatment with BRAF and

Table 2. Meta analysis exploring the impact of chemotherapy or best supportive care plus anti-EGFR monoclonal antibodies versus chemotherapy or best supportive care alone in RAS wild-type colorectal cancer.

Study (year)	Included studies (n)	Patients analyzed (n)	Line of treatment	RR (95% CI)		PFS HR (95% CI)		OSHR (95% CI)		Ref.
				RAS wt BRAF mut	RAS wt BRAF wt	RAS wt BRAF mut	RAS wt BRAF wt	RAS wt BRAF mut	RAS wt BRAF wt	
Bokemeyer *et al.* (2012)	2	845	1	1.67 (0.45–5.67)	2.27 (1.68–3.07)	0.67 (0.34–1.29)	0.64 (0.52–0.79)	0.62 (0.36–1.06)	0.84 (0.71–1.00)	[22]
Pietrantonio *et al.* (2015)	9	6256	1, 2 or more	1.31 (0.83–2.08)	–	0.88 (0.67–1.14)	–	0.91 (0.62–1.34)	–	[27]

mt: Mutated; OSHR: Overall survival hazard ratio; PFS: Progression-free survival; RR: Relative risk; wt: Wild-type.

EGFR inhibitors caused a more complete inhibition of AKT, MEK and ERK signaling as compared with vemurafenib monotherapy. Preclinical studies suggest that BRAFmt CRC might benefit from combination strategies consisting of BRAF and EGFR inhibitors or BRAF and MEK inhibitors, suppressing feedback reactivation of MAPK signaling, leading to a more robust and sustained pathway inhibition and to improved efficacy in BRAFmt CRC. Interestingly in a recent *in vitro* and *in vivo* study, Ahronian *et al.* [42] elegantly confirmed MAPK pathway reactivation as a key event in the development of acquired resistance to RAF inhibitor combinations. The authors also demonstrated that in many cases ERK inhibitors maintain their ability to suppress the MAPK pathway. Taken together, these findings suggest that ERK inhibitors could be critical components of innovation therapeutic strategies for BRAFmt mCRC, either alone or in combination with RAF and EGFR inhibitors.

BRAF targeted therapy: clinical trials

BRAF inhibition in patients with mCRC harboring BRAF mutations was clinically investigated for the first time in an extension cohort of a Phase 1 study, in which 21 previously treated patients with BRAFmt mCRC received vemurafenib (960 mg twice daily [b.i.d.]).

Table 3. Clinical trials with targeted agents in BRAF mutant metastatic colorectal cancer.

Treatment strategy	Study (year)	Phase	n	Treatment	RR (%)	PFS (months)	OS (months)	Ref.
Single agent	Kopetz *et al.* (2010)	1	18	Vemurafenib 960 mg po. b.i.d.	5	3.7	NR	[36]
	Falchook *et al.* (2012)	1	9	Dabrafenib 150 mg po. b.i.d.	11	NR	NR	[43]
	Gome-Roca *et al.* (2014)	1	18	Encorafenib 300 mg or 450 mg po. daily	0	4.0	NR	[44]
	Tabernero *et al.* (2014)†	2	10	Vemurafenib 960 mg po. b.i.d.	10	NR	NR	[45]
Double combo	Yaeger *et al.* (2015)	1/2	15	Vemurafenib 960 mg po. b.i.d. + panitumumab 6 mg/kg iv. every 14 days	13	3.2	7.6	[46]
	Tabernero *et al.* (2014)†	2	27	Vemurafenib 720 mg or 960 mg po. b.i.d. + cetuximab 300 or 400 mg as loading dose, then 200 or 250 mg weekly	7	NR	NR	[45]
	Van Geel *et al.* (2014)‡	1	26	Encorafenib 100, 200, 400 or 450 mg daily + cetuximab 400 mg/mq iv. as loading dose, then 250 mg/m^2 iv. weekly	29.2	NR	NR	[50]
	Bendell *et al.* (2014)§	1/2	17	Dabrafenib 150 mg po. b.i.d. + panitumumab 6 mg/kg iv. q14d	11.8	NR	NR	[51]
Double combo + chemo	Hong *et al.* (2014)	1b	19	Vemurafenib 480 mg po. b.i.d. , 720 mg po. b.i.d. or 960 mg po. b.i.d. + cetuximab 250 mg/m^2 q7 + irinotecan 180 mg/m^2 q14	35	7.7	NR	[52]
Triple combo	Van Geel *et al.* (2014)‡	1	25	Encorafenib 200 or 300 mg daily + cetuximab 400 mg/mq iv. as loading dose, then 250 mg/m^2 iv. weekly + alpelisib 100, 200 or 300 mg daily	30	NR	NR	[50]
	Bendell *et al.* (2014)§	1/2	16	Dabrafenib 150 mg po. b.i.d. + panitumumab 4.8 or 6 mg/kg iv. q14d + trametinib 1.5 or 2 mg	37.5	NR	NR	[51]

†In this study, vemurafenib was given as a single agent or in combination with cetuximab. Results of vemurafenib as single agent and in combination with cetuximab are reported separately in the table.
‡In this study, patients were treated with double (encorafenib + cetuximab) or triple (encorafenib + cetuximab + alelisib) combination. Results of double and triple combination are reported separately in the table.
§In this study, patients were treated with double (dabrafenib + panitumumab) or triple (dabrafenib + panitumumab + trametinib) combination. Results of double and triple combination are reported separately in the table.
b.i.d.: Twice a day; iv.: Intravenous; OS: Overall survival; PFS: Progression-free survival; po.: *Per os* (orally); q14d: Every 14 days; RR: Relative risk.

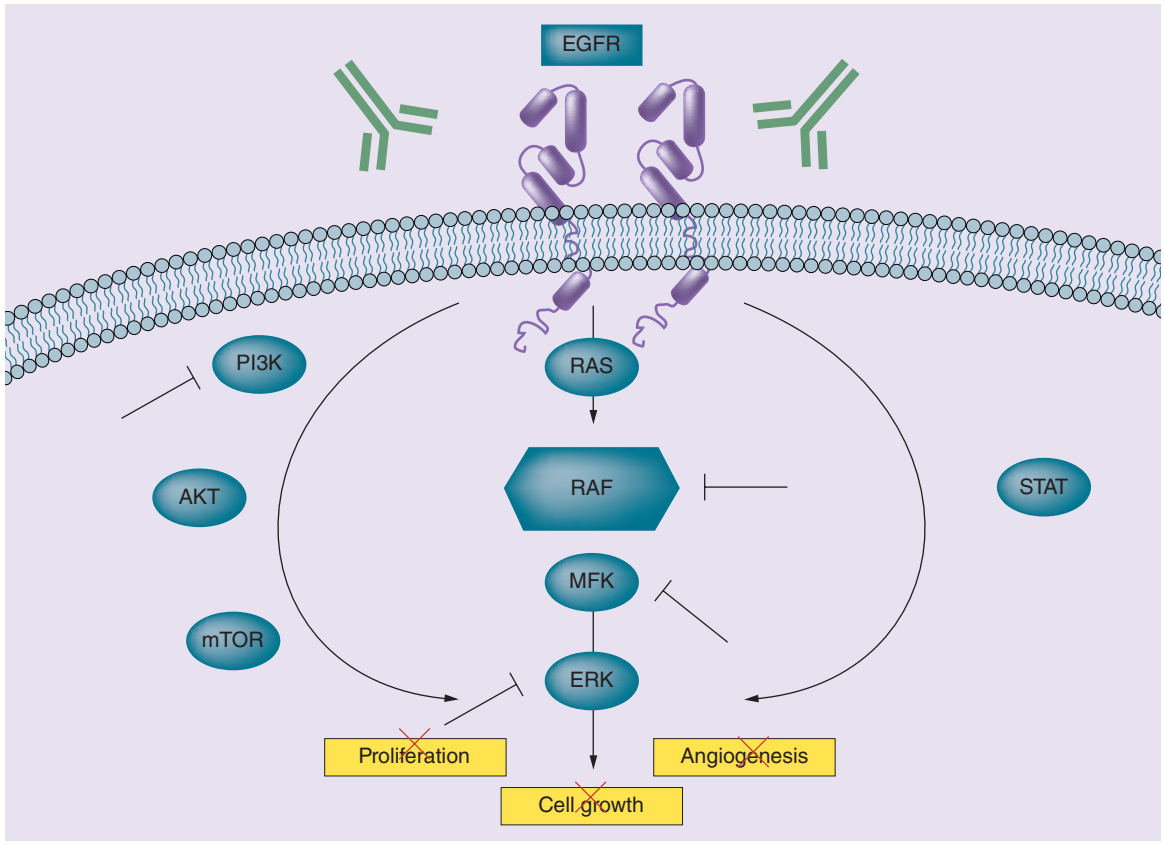

Figure 2. BRAF monotherapy inhibition and escape with collateral EGFR/MAPK signaling.

The activity of vemurafenib was far more modest than in BRAFmt melanoma, with only one confirmed partial response reported [36] (Table 3). Grade 3–4 adverse events included fatigue, skin rash, diarrhea, neutropenia, elevated alkaline phosphatase, electrolytes disorder and hyperbilirubinemia; five patients developed cutaneous squamous cell carcinomas. Similarly, limited activity was reported in different studies with BRAF inhibitors used as single agent [43–45] (Table 3). The disappointing results achieved by single-agent BRAF inhibition, might be explained by the rapid restoration of ERK activity in BRAFmt CRC due to feedback activation of EGFR/MAPK pathway (Figure 2). This evidence, shown by preclinical models, provided the biological rationale for investigating the dual blockade of EGFR and BRAF [37,39]. In a pilot trial, 15 patients with BRAFmt mCRC, progressed through at least one previous treatment, received a combination of vemurafenib (960 mg b.i.d.) and panitumumab (6 mg/kg every 14 days) (Table 3). Two patients (13%) achieved a partial response and other two patients had stable disease lasting over 6 months [46]. The combination therapy was overall well tolerated. Acneiform rash and fatigue, primarily grade 1, were the most frequently observed adverse events. Interestingly, incidence and severity of acneiform rash was lower than that observed with vemurafenib and panitumumab used as single agents [47,48]. This finding is probably due to the different effect of the two drugs on ERK signaling. In fact, in BRAF wild-type cells such as epidermal keratinocytes, vemurafenib stabilizes the active dimeric conformation of BRAF resulting in increased ERK signaling [49], and this counteracts the ERK inhibition secondary to EGFR blockade induced by panitumumab, leading to decreased cutaneous adverse events. On the other hand, the trial reported an increased toxicity in terms of abnormalities of liver function tests, with 20% of patients experiencing grade 3 alkaline phosphatase elevation and two patients grade 4 transaminase increase [46]. The activity of EGFR and BRAF dual blockade observed in this study was consistent with that reported in other similar studies. In a Phase 2, Simon 2-stage adaptive design study in non-melanoma BRAFmt patients, vemurafenib (740 or 960 mg b.i.d.) was given in combination with cetuximab (300 mg/m^2 as loading dose, then 200 mg/m^2 weekly; or 400 mg/m^2 as loading dose, then 250 mg/m^2 weekly) to 27 patients with mCRC. Preliminary results reported a partial response in 2 (7%) patients and a disease stabilization in 14 (52%) patients with a clinical benefit reported for at least 20% (4 out of 21) of patients in the cohort treated with both drugs at the full dose [45] (Table 3). Safety profile of the combination was

Table 4. Ongoing clinical trials.

ClinicalTrials.gov identifier	Drugs	Notes
NCT02175654	Regorafenib[†,‡]	Phase 2, second-line study in mCRC patients with any RAS or BRAF mutation progressed after FOLFOXIRI + bevacizumab regimen
NCT01719380[§§]	Encorafenib[†] + cetuximab[§] Encorafenib[†] + alpelisib[¶] + cetuximab[§]	Phase 1–2 study in patients with BRAFmt mCRC
NCT02164916	Cetuxumab[§] + Irinotecan[#] +/- Vemurafenib[†]	Phase 2 randomized trials in BRAFmt mCRC patients
NCT01750918	Dabrafenib[†], trametenib[††], panitumumab[§]	Four part, Phase 1–2 study. Part 1: dose-escalation cohorts Part 2: expansion cohorts with dabrafenib + panitumumab and trametinib + dabrafenib in combination with panitumumab Part 3: randomized Phase II study comparing dabrafenib + panitumumab andtrametinib + dabrafenib + panitumumab versus standard chemotherapy with or without panitumumab or bevacizumab Part 4: investigation of trametinib + panitumumab in two patient populations: BRAFmt CRC and subjects with CRC who developed secondary resistance to prior anti-EGFR therapy
NCT01877811	CEP-32496[††]	Phase 1–2, points with BRAFmt melanoma or mCRC
NCT02278133	WNT974[‡‡], encorafenib[†], cetuximab[§]	Phase 1b-2, points with BRAFmt mCRC
NCT01116271	Selumetinib[††] + irinotecan[†]	Dose finding Phase 2, second-line study in points with RAS or BRAF mutation after oxaliplatin-based first-line therapy
NCT01086267	XL281[†] + cetuximab[§]	Phase 1–2, RAS or BRAFmt CRC

Class of drugs:
[†]BRAF inhibitor.
[‡]Mutlikinase inhibitor.
[§]Anti-EGFR antibody.
[¶]PI3K inhibitor.
[#]Chemotherapy.
[††]MEK inhibitor.
[‡‡]PORCN inhibitor.
[§§]Preliminary results already reported (Van Geel *et al.* 2014).
CRC: Colorectal cancer; mCRC: Metastatic colorectal cancer; mt: Mutated.

manageable, and the most frequently observed adverse events were diarrhea, arthralgia and rash (63% for each event). Notably, once again liver toxicity was of some concern, since one of the two patients who discontinued treatment because of adverse events developed grade 2 hepatic degradation and grade 3 hyperbilirubinemia, whereas the other patient had a grade 3 gastrointestinal bleeding. Furthermore, a treatment-related death was reported, due to multifocal progressive pneumonitis. In another Phase 1 study 51 patients received cetuximab (400 mg/m^2 as loading dose, then 250 mg/m^2 weekly) in combination with encorafenib at increasing dose (100, 200, 400 or 450 mg *per os* [orally; po.] daily) in the first part of the study (dual combination), and then cetuximab plus encorafenib (200 or 300 mg po. daily) in combination with the PI3K inhibitor alpelisib (also named BYL719) at increasing dose (100, 200 and 300 mg po. daily) in the second part of the study (triple combination) [50] (Table 3). Response rate was 30% for both the dual and the triple combination, and DCR was about 80% for the dual combination and 90% for the triple combination. The most common treatment-related adverse events were fatigue and nausea in the dual combination group, and nausea and acneiform dermatitis in the triple combination group. The dual blockade of BRAF and EGFR with dabrafenib and panitumumab was evaluated alone, or in combination with the MEK inhibitor trametinib at different dose levels in a Phase 1–2 study on BRAFmt mCRC patients. Preliminary efficacy results showed 2 partial responses in the dual blockade group (n = 15) and 1 complete response and 5 partial responses in the triple blockade group (n = 16) [51] (Table 3). The triple combination was able to provide a more robust inhibition of ERK than the dual combination, and this might have contributed to higher clinical activity. The triple combination with each drug at its standard dose as single agent was well tolerated, with no grade 4 adverse events reported. The

addition of chemotherapy to dual BRAF and EGFR inhibition was investigated in a Phase 1b study. Patients with BRAFmt mCRC were treated with irinotecan (180 mg/m^2 every 14 days), cetuximab (250 mg/m^2 weekly) and escalating doses of vemurafenib (480, 720 and 960 mg b.i.d.). Maximum tolerated dose of vemurafenib was 960 mg, and the most common adverse events were fatigue (94%), diarrhea (89%), nausea (83%) and rash (78%). A partial response was reported in 6 (35%) of 17 evaluable patients, and median PFS was 7.7 months [52] (Table 3). On the basis of these encouraging results, a US cooperative group randomized Phase 2 trial of irinotecan and cetuximab with or without vemurafenib in BRAFmt mCRC is ongoing (SWOG 1406) (Table 4). In conclusion, BRAF inhibitors as single agent have no meaningful activity in BRAFmt mCRC. Dual combination of BRAF inhibitors and anti-EGFR agents, with or without chemotherapy, showed promising activity, with response rates ranging from 10 to 30%. Triple combination of BRAF inhibitors plus anti-EGFR agents with MEK or PI3K inhibitors might further improve the activity of target therapy for BRAFmt mCRC patients (Figure 3). Combinations of targeted agents are well tolerated with fatigue, arthralgia, diarrhea and rash representing the more frequently reported adverse events. Cutaneous toxicity typical of BRAF inhibitors and anti-EGFR antibodies as single agents is mitigated when the two drugs are combined together, probably due to their opposing activity on skin. Liver toxicity might raise some concern in mCRC patients who have hepatic metastases in the majority of cases, with an increased risk for liver dysfunction. Several clinical trials are currently ongoing to further investigate efficacy and safety of BRAF inhibitors in combination with chemotherapy or other targeted agents (Table 4).

BRAF, MSI & immunotherapy

MSI is a biological consequence of a germline mutation of one of the mismatch repair (MMR) genes in hereditary nonpolyposis colon cancer whereas it is mostly linked to hMLH1 promoter methylation in sporadic tumors [15,53–60]. In MSI-high tumors the proportion of BRAF mutations ranges from 13 to 78% [61,62]. On the contrary in microsatellite stable tumors BRAF mutations are detected in less than 10% [15,53–57]. Recently a Phase 2 study assessed the clinical activity of pembrulizumab (10 mg/kg every 2 weeks), a humanized monoclonal antibody that blocks the interaction between PD-1 and its ligands PD-L1 and PD-L2, in patients with previously treated, progressive metastatic disease with or without MMR-deficiency [63]. Three groups were evaluated: MMR-deficient mCRC (n = 13),

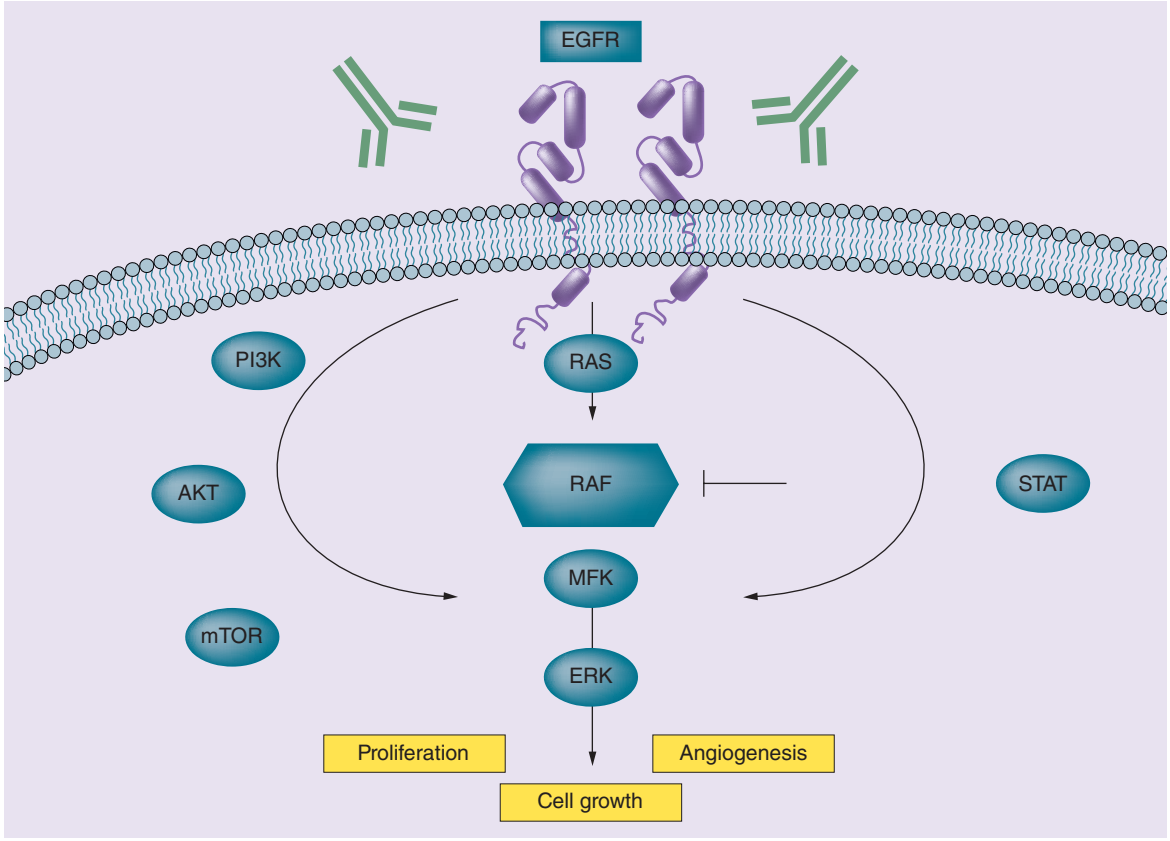

Figure 3. Multiple EGFR/BRAF inhibition in colorectal cancer cell.

MMR-proficient mCRC (n = 25) and MMR-deficient other cancers (n = 10). MMR status was assessed using a standard polymerase chain reaction-based method for detection of MSI. The primary end points of the study were immune-related PFS rate assessed at 20 weeks and ORR; secondary end points included OS, PFS and DCR. In the MMR-deficient mCRC subgroup the ORR was 62% and the DCR was 92%. No responses were observed in the MMR-proficient mCRC and the DCR was 16%. In the MMR-deficient other cancers group, the ORR was 60% and DCR was 70%. Median PFS and OS were not reached in the MMR-deficient mCRC group, whereas in the MMR-proficient mCRC group PFS was 2.3 months and OS was 7.6 months. The median duration of follow-up for all patients was 5.9 months (0.9–16.6 months): 8.3 months (2.2–16.6 months) in the MMR-deficient mCRC group, 4.9 months (0.9–15.6 months) in the MMR-proficient mCRC and 7.1 months (2.4–16.4 months) in the MMR-deficient other cancers group. Of all responders, no patients in the MMR-deficient CRC group and one patient in MMR-deficient other cancers group had progressed at the time of the analysis. Given the efficacy of this treatment in patients with MRR-deficient mCRC and the close link between MSI and BRAF mutations, anti-PD-1 immunotherapy might represent an interesting option in a consistent subgroup of BRAFmt mCRC.

Conclusion & future perspective

Patients affected by BRAFmt mCRC experience a rapidly progressive disease with poor prognosis. However, more recently, we showed that BRAF codon 594 or 596 mutated mCRCs are different from BRAF V600E ones in terms of molecular features, pathological characteristics and clinical outcome [64]. This is consistent with preclinical evidences of a kinase inactivating effect of these mutations and may be the rationale for specific targeted treatment.

Anti-EGFR therapy showed no significant efficacy in this setting [65]. Moreover, given the biological aggressiveness of this disease, patients barely reach following treatment lines. Therefore, intensive, though more toxic, chemotherapy combination regimen (FOLFOXIRI ± bevacizumab) appears nowadays the most suitable therapeutic option in this group of patients. BRAF inhibitors monotherapy appeared ineffective due to the redundant role of EGFR pathway in mCRC. However, slow significant progress in understanding BRAFmt mCRC has been made in preclinical studies. The efficacy of BRAF and EGFR inhibitors combination in preclinical models and in clinical trials, have generated great optimism that effective targeted therapy might soon be available for patients with BRAFmt mCRC. Immunotherapy (PD-1 blockade) seems to represent an interesting option in mCRC with MSI, frequently associated with BRAF mutations. Whether the efficacy of these new strategies would be confirmed in prospective Phase III trials, the prognosis of patients with BRAFmt mCRC could dramatically change and this currently treatment orphan disease could benefit from efficient treatment.

Executive summary

Biological background

- BRAFmt are detectable in about 5–15% of metastatic colorectal cancer (mCRC) patients and represent a clear negative prognostic factor.
- BRAFmt occur in two regions of the BRAF kinase domain, the glycine-rich loop and the activations segment. Although several BRAF mutations were evaluated in many studies, the biological effects of each mutations were not fully understood.
- In CRC cell selective BRAF inhibition determine a feedback reactivation of MAPK signaling by EGFR-mediated activation of RAS and CRAF. Melanoma cells, deriving from the neural crest, express low levels of EGFR (expressed primarily in epithelial cancers) and are therefore not subject to this feedback activation.

Present clinical implication

- BRAFmt seems a predictor of resistance to anti-EGFR monoclonal antibody-based therapy, thus BRAF mutational status assessment should be performed before initiation of treatment with anti-EGFR monoclonal antibodies.
- Nowadays intensive chemotherapy combination regimen such as FOLFOXIRI ± bevacizumab appears the most suitable therapeutic option in BRAFmt mCRC.

Future perspective

- BRAFmt mCRC patients might benefit from combination strategies of BRAF and EGFR inhibitors or BRAF and MEK inhibitors, suppressing feedback reactivation of MAPK signaling.
- ERK inhibitors seem to maintain their ability to suppress the MAPK pathway reactivation, therefore ERK inhibitors could be critical components of innovation therapeutic strategies for BRAFmt mCRC, either alone or in combination with BRAF and EGFR inhibitors.
- Anti-PD-1 immunotherapy might represent an interesting option in BRAFmt MRR-deficient mCRC.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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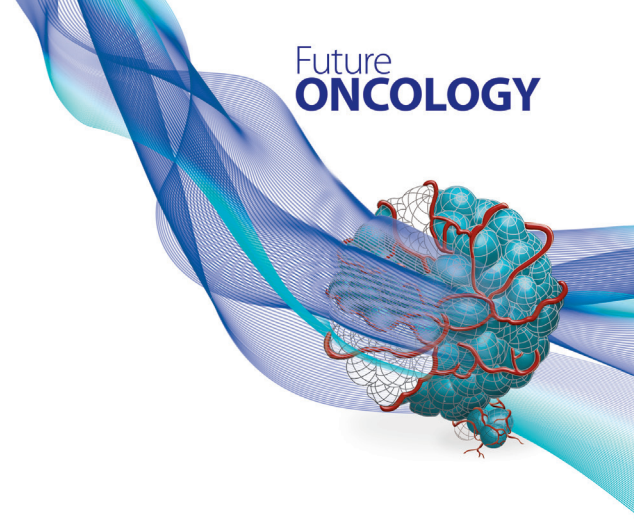

Colorectal clinical trials: what is on the horizon?

Daniel H Ahn[1] & Richard M Goldberg*[,1]

Substantial progress has been made in the treatment of colorectal cancer, where more effective therapies have led to improved outcomes in patients with advanced disease. However, the 5-year overall survival rate remains poor. Genomic sequencing has allowed us to understand that colorectal cancer is a heterogeneous disease, where tumor-specific variants affect the prognosis and outcomes in patients. This has shaped the future directions of treatment and the development of clinical trials, including the incorporation of novel targeted therapies and investigations into the role of immunotherapy in colorectal cancer.

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KEYWORDS
• colorectal cancer • immunotherapy • next-generation sequencing • targeted therapy

Colorectal cancer (CRC) is the fourth most common cancer among men and women (when skin cancer is included) and is the second leading cause of cancer-related deaths in the USA [1–3]. Proactive measures, including screening colonoscopies and fecal occult blood testing, have allowed the early identification of premalignant polyps and CRC in its early and curable stages. A diagnosis of stage I–III CRC allows more patients to undergo treatment with a curative intent. However, a great proportion of patients are diagnosed with metastatic disease. Significant progress has been made over the past two decades in the treatment of metastatic CRC with the duration of the median overall survival increasing from approximately 12 months to nearly 30 months [4–6]. This has been in large part due to a greater understanding that CRC is driven by a variety of genetically heterogeneous mutations. This realization is leading to an improvement in patient selection for treatment with selected targeted treatment regimens. However, the 5-year survival rate of metastatic (mCRC) remains <12.5%, highlighting the need for the development of new agents and new approaches to treatment [2]. With an increased understanding, clinical trials are now being developed to assign therapy based upon specific tumor features identified principally by analysis of the individual tumor's genomic changes and targeting these mutations with novel therapeutic agents. Herein, we will discuss future directions in the treatment for advanced or metastatic CRC.

Immunotherapeutic approaches in mCRC

• The role of checkpoint inhibitors in the treatment of colorectal cancer

With the exception of the recent development of specific treatments for certain solid tumor malignancies (renal cell carcinoma, melanoma, prostate cancer and non-small-cell lung cancer), immunotherapy has been extensively tested yet it has remained experimental. Tumors evade a host immune response by dampening the response of tumor-specific T cells and by expressing ligands that bind to

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inhibitory receptors or 'checkpoints' that decrease the innate antitumor immune response [7,8]. Using pharmacologic interventions to disable these checkpoints can enhance pre-existing anticancer immune responses. Immunotherapies targeting negative regulatory molecules on activated T cells, including CTLA-4, PD-1 and its binding ligand PD-L1 have shown promising antitumor activity in several malignancies. While initial studies in CRC with these immunotherapeutic agents have been disappointing [9,10], findings suggest that selected patients with CRC may benefit from immunotherapy. A Phase I study conducted by Brahmer *et al.* demonstrated a sustained complete response (>21 months) in one patient with mCRC treated with MDX-1106, a PD-1 inhibitor [10]. The patient's tumor was found to lack expression of mismatch repair proteins and thus was classified as MSI-H or having a high degree of microsatellite instability. Previous studies demonstrated that mismatch repair-deficient cancers contain prominent Crohn's disease-like lymphocyte infiltrates suggesting that these tumors elicit an innate immune response [11–14]. In addition, initial studies indicated antitumor activity with immunotherapeutic agents in tumors with high rates of somatic mutations. Because of these findings, a single-arm Phase II study was conducted to investigate the clinical activity of pembrolizumab, a PD-1 inhibitor, in patients with progressive metastatic carcinoma with or without mismatch-repair deficiency [15]. Patients with mismatch repair-deficient CRC demonstrated an objective response rate of 40% and immune-related progression-free survival of 78% at 20 weeks. Interestingly, no immune-related objective response was seen in mismatch repair-proficient CRC patients, confirming that immunotherapy may be beneficial in only certain subsets of CRC unless additional strategies can be developed to render those tumors to be more immunogenic [15]. Based on this promising clinical activity, several ongoing studies are investigating various immunotherapeutic agents in the treatment of CRC, including trials in patients with microsatellite instability high tumors and those with high levels of PD-1 expression **(Table 1)**. While ongoing studies will assess and confirm its clinical utility in a subset of mCRC patients, an understanding in mechanisms of resistance, duration of required therapy and predictive biomarkers of response are needed.

• Cancer vaccine therapies

Cancer vaccine therapies are an attractive potential therapeutic approach as they have the potential to trigger the immune system to respond to tumor-specific antigens and attack cancer cells. Several types of vaccinations are under investigation against CRC and include DNA, viral, peptide and tumor cell vaccines.

• GVAX

GVAX is an irradiated whole-cell-modified vaccine composed of autologous irradiated colon cancer cell lines engineered to express granulocyte-macrophage colony stimulating factor. Granulocyte-macrophage colony stimulating factor plays a vital role in stimulating the immune system response by inducing dendritic cell differentiation. Several studies investigating the immunologic effects of GVAX have demonstrated its ability to create an inflammatory reaction causing an upregulation of PD-L1. This finding suggests the potential utility of combining this vaccine with immune checkpoint inhibitors [16,17]. GVAX is currently being investigated with the combination of SGI-110, a DNA hypomethylating agent and cyclophosphamide in mCRC (NCT01966289).

• Peptide vaccines

Peptide vaccines employ an eight to 11 amino acid epitope of an antigen that is recognized by effector T cells. This approach is based on the identification and synthesis of epitopes, which can

Table 1. A highlight of ongoing immunotherapy trials for colorectal cancer.

Agent	Class of agent	Trial number	Phase	Comment
MK-3475	Anti-PD-1	NCT01876511	II	MSI-high tumors
MEDI4736	Anti-PD-L1	NCT01693562	I/II	
Nivolumab ± ipiliumumab	Anti-PD-1/anti-CTLA-4	NCT02060188	I/II	Recurrent and metastatic CRC
MK-3475 + mFOLFOX6	Anti-PD-1	NCT02375672	II	
Tremelimumab + MEDI4736	Anti-CTLA-4 + anti-PD-L1	NCT01975831	I	

CRC: Colorectal cancer; MSI: Microsatellite instability.

Table 2. A highlight of ongoing signaling pathway inhibitor trials for colorectal cancer.

Agent	Class of agent	Trial number	Phase	Comment
MEK162 + panitumumab	*MEK* tyrosine kinase inhibitor, anti-EGFR mAb	NCT01927341	Ib/II	mCRC with mutant or wild-type *RAS* tumors
Dabrafenib + trametinib + panitumumab + 5-fluorouracil	*BRAF* tyrosine kinase inhibitor, MEK tyrosine kinase inhibitor, anti-EGFR mAb	NCT01750918	I/II	*BRAF*-mutation V600E + and in patients with secondary resistance to anti-EGFR mAb
LGX818 + cetuximab ± BYL719	*BRAF* tyrosine kinase inhibitor, anti-EGFR mAb, PI3K tyrosine kinase inhibitor	NCT01719380	I/II	*BRAF*-mutant mCRC
Irinotecan + cetuximab ± vemurafenib	anti-EGFR mAb, *BRAF* tyrosine kinase inhibitor	NCT02164916	II	*BRAF*-mutant mCRC
Neratinib + cetuximab	*HER2* tyrosine kinase inhibitor, anti-EGFR mAb	NCT01960023	I/II	*KRAS*, *NRAS*, *BRAF*, *PIK3CA* wild-type

mAb: Monoclonal antibody; mCRC: Metastatic colorectal cancer.

induce tumor antigen-specific immune responses. Since these agents are derived from tumor-specific antigens, they have a decreased risk of inducing autoimmunity. Several peptide vaccines for CRC have reached Phase I trials, demonstrating promising signs of clinical activity [18,19]. With HER2 overexpression present in a proportion of CRC [20,21], HER2 peptide vaccines and their potential roles as a therapeutic agent in CRC are currently being investigated (NCT01376505).

• Oncolytic viral therapy

Given their tumor selectivity and ability to induce cancer cell lysis, oncolytic viral therapy represents an area of interest in cancer treatment. Through alterations induced in their genetic structure, these viruses target and lead to the destruction of cancer cells, and through additional alterations, prevent the binding and replication of the virus in normal, healthy cells. Reovirus is a family of naturally occurring, nonenveloped human virus whose replication is dependent upon the cellular activity of *RAS*. Specifically, it is cytopathic in transformed cells possessing an activated *RAS* signaling pathway [22–25]. Given the prevalence of *K-RAS* and *N-RAS* mutations in CRC, the use of reovirus has represented a promising and attractive candidate as an oncolytic virus in this disease. It is currently being investigated in combination with FOLFIRI and bevacizumab in *K-RAS* mutant metastatic colorectal cancer (NCT01274624).

Targeting relevant downstream signaling pathways in mCRC

Targeting signaling pathways remains an attractive therapeutic strategy in CRC. Given the high presence of mutations in the oncogene *RAS* (*KRAS* and *N-RAS*) in CRC and its role on cell survival and proliferation, targeting *RAS* represents a promising strategy. While its role as a predictive biomarker in anti-*EGFR* therapy has been established, its relevance as a therapeutic target remains undefined. Targeting *RAS* mutations directly has remained a challenge. An alternative approach has been to inhibit downstream effector pathways of the *MAPK* pathway (e.g., *BRAF*, *MEK*). The clinical activity is often short-lived, due to compensatory mechanisms that include cross-talk between parallel downstream signaling pathways, downstream activation and negative loop feedback inhibition, and the development of treatment resistance [26,27].

Alternative strategies against *RAS*-mutant mCRC include combining agents against multiple signals of the *MAPK* pathway to cause sufficient inhibition of *RAS* activity, where preliminary findings have demonstrated promising clinical activity [28]. The combination of *MEK* and *EGFR* inhibitors have demonstrated the reversal of acquired anti-*EGFR* resistance when *MEK* inhibition is added to therapy [29,30], which has prompted the development of clinical trials investigating combination of signaling pathway inhibitors as a primary therapeutic option and as salvage therapies in the refractory disease setting (Table 2). Additionally, targeting multiple signaling pathways may be an effective treatment strategy to overcome resistance of secondary activation of parallel signaling pathways, including studies investigating the concurrent inhibition of the *PI3K* and *MAPK* pathway [31].

Mutations of the oncogene *BRAF* are present in approximately 5–10% of mCRC [32,33]. Patients with mCRC whose tumors harbor *BRAF* V600 mutations generally respond poorly to conventional systemic therapies and are associated with poor outcomes [34–39]. *BRAF* inhibition with

BRAF small molecule inhibitors (vemurafenib or dabrafenib) has led to improve outcomes in progression-free survival and overall survival in patients with *BRAF*-mutated melanoma [40–43]. However, in contrast to melanoma, mCRC with *BRAF* V600 mutations have not shown similar efficacy, with a lack of sensitivity to *BRAF* inhibitor monotherapy [42,44]. One rationale for the lack of clinical activity in *BRAF*-mutant CRC may be due to insufficient blockade of the *MAPK* pathway due to a compensatory feedback loop mechanism, leading to reactivation of the pathway **(Figure 1)** [27,45]. The combination of multiple inhibitors of the *MAPK* pathway has demonstrated significant improvement in patient outcomes in metastatic *BRAF* V600-mutated melanoma [46]. Based on these findings, a recent Phase II study by Corcoran *et al.* investigated the clinical efficacy of combined *BRAF* and *MEK* inhibition with dabrafenib and trametinib in patients with *BRAF*-mutant mCRC [47]. The findings overall were disappointing, where only 12% of patients experienced a partial or complete response and 56% had stable disease. Correlative studies demonstrated *MAPK* signaling inhibition but to a lesser degree that was observed in *BRAF*-mutant melanoma treated with dabrafenib [43]. One rationale for the lack of activity may be due to inadequate *MAPK* signaling inhibition. Preclinical studies have suggested that *EGFR* may contribute to overcoming *BRAF* inhibition, leading to reactivation of the *MAPK* and other key signaling pathways [26]. Ongoing clinical trials are evaluating the combination of *EGFR* monoclonal antibodies with *BRAF* inhibitors [48–51] in *BRAF*-mutant mCRC.

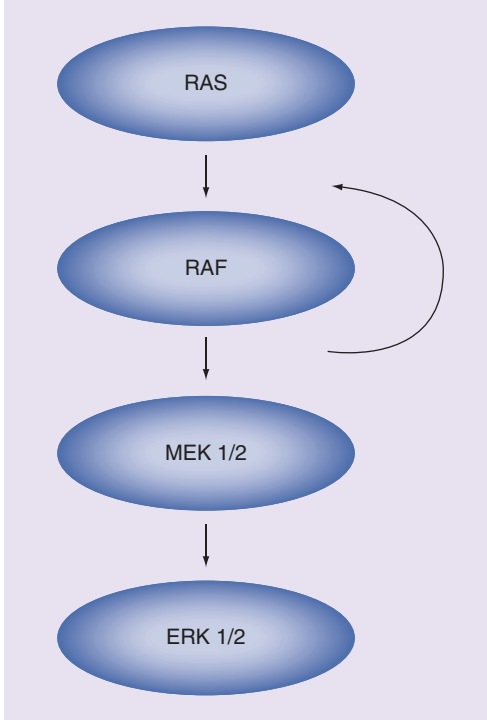

Figure 1. RAS/RAF/MEK/ERK pathway.

In patients whose tumors do not express mutations in *RAS*, antiangiogenic and targeted agents against *EGFR* have demonstrated a clinical benefit in mCRC [52–61]. Ongoing studies in this patient population include strategies targeting both *VEGF* and *EGFR* that include combining cetuximab and bevacizumab with chemotherapy (ClinicalTrials.gov NCT00265850) and cabozantinib, a multi-target (VEGFR2, MET) small molecule inhibitor with panitumumab (CaboMab trial, ClinicalTrials.gov NCT02008383).

• Molecular profiling, heterogeneity & personalized therapies with targeted agents against signaling pathways in CRC

Through the efforts by the Cancer Genome Atlas Network, we have a better understanding of the genomic alterations present in CRC which has allowed us to identify potential therapeutic targets in CRC [62]. A total of 224 CRCs underwent comprehensive molecular characterization, where several mutated genes were considered relevant targets for treatment. *HER-2*, *ROS-1*, *ALK* fusion and *c-MET* overexpression were among the identified mutations in a small proportion of CRC [63,64]. This increased understanding of the genomic alterations in CRC in addition to the availability of next-generation sequencing has allowed development of 'personalized' therapies through clinical trials investigating genomic mutations of interest.

Conclusion & future perspective

With the incorporation of combination cytotoxic chemotherapy and targeted therapies into the treatment for mCRC, patient outcomes have been progressively improving over the past two decades. However, the prospect for long-term survival and the prognosis remains poor, with a subset of patients surviving less than 1 year. Advancements in genomic sequencing have led to a new understanding that CRC is a heterogeneous disease, where tumor-specific variants significantly affect the prognosis and outcomes in patients. Incorporation of molecular profiling can direct the development of clinical

trials, allowing treatment arms to be tailored to individual tumor-specific genomic alterations.

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EXECUTIVE SUMMARY

- The role for immunotherapy in colorectal cancer (CRC) remains undefined but specific interventions appear to benefit subsets of patients.
- Immunotherapy may be beneficial in selected patients with CRC, notably those with somatic mutations, including microsatellite instability high tumors that are hypermutated and thus present more antigens for potential targets.
- Confirmatory studies are investigating the role of immunotherapy in selected CRC and attempting to identify predictive biomarkers for response.
- Vaccine therapies remain a promising but experimental therapeutic approach in the treatment of CRC.
- Antitumor activity from signaling pathway inhibition is short-lived due to multiple mechanisms for resistance.
- Ongoing strategies are investigating the role of multiple pathway inhibition.
- Next-generation sequencing has demonstrated that a small proportion of CRC have genomic alterations that are of therapeutic interest.

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REVIEW

Colorectal Cancer

Biologic and molecular markers for staging colon carcinoma

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Practice points

- MSI-H CRC show more favorable prognosis than MSS or MSI-L CRC.
- MSI-H CRC patients do not benefit from 5-fluorouracil-based adjuvant chemotherapy.
- MSI-H CRC with loss of expression of MSH2, MSH6 and PMS2 are strongly associated to Lynch syndrome.
- *KRAS* mutation in CRC is a predictor of resistance to therapy with anti-EGFR therapy (panitumumab or cetuximab) and is associated to shorter free-disease progression.
- *NRAS* mutation in CRC is a predictor of no benefit with anti-EGFR therapy.
- *BRAF* mutation in CRC plays a strong negative prognostic role, is a predictor of resistance to anti-EGFR therapy and is associated to shorter survival.
- *BRAF* mutation is almost mutually exclusive with *KRAS* mutation.
- *BRAF* V600E mutation in MSI CRC almost always excludes Lynch syndrome.
- *PIK3CA* mutation in CRC is associated to poor prognosis.
- CIMP-H CRC do not benefit from 5-FU-based adjuvant chemotherapy.
- CIMP-H tumors are associated with *BRAF* mutation while CIMP-L tumors are associated with *KRAS* mutation.

Biomarkers in the field of pathology and oncology may allow for the detection of disease, assessment of prognosis or to predict response to certain therapy. Molecular abnormalities in colorectal cancer genesis may occur due to chromosome instability, microsatellite instability and DNA methylation (CpG island methylator phenotype). These alterations are associated in some cases to sporadic carcinomas whereas in others are seen in syndrome-related tumors and are the basis for the use of different biomarkers in the clinical setting. These may include mismatched repair gene/proteins, RAS, BRAF, PIK3CA, which help to determine tumor prognosis and predict response to certain drugs.

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Colorectal adenocarcinoma is the second and third most frequent type of cancer diagnosed among men and women respectively worldwide. In developed countries, it is the second cause of mortality from cancer in men and the third in women [1]. Until recently, the progression, diagnosis, therapeutic approaches and prognostic indicators were mostly based on the anatomical extension reflected by the depth of invasion of the tumor (pT) into the wall of the colon or rectum and the presence

KEYWORDS

• biomarkers • chromosome instability • colorectal cancer • DNA methylation • Lynch syndrome • mismatch repair proteins

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Future Medicine part of fsg

or absence of lymph node metastasis (pN). In those cases without nodal metastasis (stage II), several histopathological factors associated with adverse outcome, including vascular and perineural invasion, poor tumor differentiation and an infiltrative pattern of tumor growth at the edge of the tumor in the form of single cells or small clusters of cells (budding) have been applied to predict prognosis and decide on adjuvant therapy. Most of these parameters, however suffer from inter and intraobserver variability. The ongoing broadening knowledge of the molecular characteristics of colorectal neoplasia has initiated an era of cancer treatment focused on the individual profile of each tumor. This individuality calls for selection of specific first-line adjuvant pharmacological therapy [2,3]. The molecular heterogeneity of colorectal carcinoma not only influences the behavior and response to certain drugs, but also makes it clear that the use of molecular signature markers is now mandated to complement the tumor evaluation as morphology alone and current staging system at examining the resected tumor specimen does not show the entire picture. For instance, a percentage of patients thought to be at early stage develop aggressive disease. Attributes for a more aggressive behavior have included involvement of serosa, and vascular and perineural invasion [4,5]. The sensitivity or resistance to certain drugs can now be predicted as is the case of patients whose tumors harbor *KRAS* mutations making them unsuitable to be treated with agents that target EGFR [6,7]. Likewise, there are tumors that may show microsatellite instability (MSI), which may be resistant to the use of 5-FU [8]. Also, molecular markers are able to determine if a carcinoma is sporadic in a given patient, as it occurs in about 80% of cases, or if it has been the result of a genetic predisposition with implications for the patient's blood-line relatives as in the Lynch syndrome [9].

Many of these achievements have been possible through a more detailed understanding of colorectal cancer carcinogenesis. A brief review of the different pathways is offered in the next section.

Molecular pathways in colorectal carcinoma

There are three distinct molecular pathways known to play a role in the development of colorectal carcinoma. They include chromosome instability, MSI and DNA methylation (CpG island methylator phenotype [CIMP]). The carcinogenesis pathways for CRC are not mutually exclusive. Thus, a carcinoma can exhibit features of multiple pathways [10–12].

• Chromosomal instability

This is the traditional pathway, originally described by Morson [13] that results from an accumulation of chromosomal abnormalities including gains, losses and translocations in oncogenes and tumor suppressor genes which lead to aneuploidy. Malignancies that arise following this track originate from adenomas that gain successive chromosomal aberrations (adenoma-carcinoma sequence). This pathway of aneuploidy is responsible for about 70% of colorectal carcinomas. Typically, mutations in the *APC-β catenin* cascade and *K-ras* genes are early events in the neoplastic process whereas abnormalities in the *TP53* and *SMAD4* occur later [14].

The genetic predisposition for malignancies of some syndromatic individuals led to the discovery and their repercussions of genes that play a role in colorectal carcinoma not only in high-risk families but in sporadic carcinomas as well.

The adenoma-carcinoma sequence

Most colonic carcinomas arise from adenomas [15]. This transformation described initially by Morson [13] is due to a multistep genome instability [16] in which there is increased acquisition and tolerance of mutations in suppressor genes and oncogenes leading to various pathways that end up in the carcinoma formation. There appears that the presence of aberrant crypt foci is an intermediate step between normal colonic epithelium and the development of adenoma [17].

• Colorectal carcinoma genes & syndromes: familial adenomatous polyposis

Familial adenomatous polyposis (FAP) is an autosomic dominant disorder that affects women and men equally, the gene responsible for this condition was identified in 1987. The patients are usually under 40 years of age and their colons show hundreds of adenomatous polyps. Essentially all patients with FAP will develop carcinoma in their colons if untreated and they represent less than 1% of all CRCs. Other portions of the GI tract are affected in particular stomach and duodenum. The germline mutations of the *APC* tumor suppressor gene in these conditions occur on chromosome *5q21* negatively regulating the Wnt/Wingless pathway [18]. The final result is

activation of proto-oncongenes such as *c-myc*, increase in the cellular proliferation and reduction in apoptosis [14,19]. Currently, there are more than 700 mutations known whose location in the *APC* gene influence the severity and body site manifestations. The diagnosis is confirmed by screening or initial testing by PCR, Southern blot or FISH followed by DNA sequencing.

• Polyposis syndrome associated to MUTHY

Clinically, it is similar to FAP including the presence of colonic and duodenal adenomas. However, it is a recessive autosomal disorder and almost all homozygous patients for this mutation develop colorectal carcinoma. Instead of mutations in the *APC* gene, these patients carry mutations in the *MUTYH* gene located on chromosome 1p, which encodes a DNA glycosylase in charge of excising bases that have been altered thus, preventing further mutations in other genes. If altered, there will be increased G:C to T:A transversions [20]). Two mutations, Tyr165Cys and Gly382Asp, have clinical relevance and are identified by gene sequencing [21].

• CpG island methylator phenotype

The majority of colorectal cancers are the result of genetic alterations that result from DNA abnormalities. However, it has been shown that a group of malignancies are the consequence of the so called 'epigenetic changes,' which refers to the fact that the aberrations within specific genes occur after the DNA duplication is completed. It is characterized by a set of gene-associated CpG islands. The methylation of certain genes (*MLH1*, *CDKN2A*, *p16*, *MGMT*, *IGF2*, *RUNX3*, *SOC51*, *MINTs* and others), in particular tumor suppressor genes is one of the main epigenetic mechanisms in colon carcinoma [22,23]. For instance, inactivation of the MLH1 by hypermethylation causes defective mismatch repair in sporadic colorectal carcinomas with some of these being MSI-H but are not associated with Lynch syndrome. These tumors frequently show mutations in the *BRAF* gene and CpG island methylation, are known as CpG island methylator phenotype (CIMP- H) and display extensive levels of methylation genes. These cases are identified by PCR and are more commonly encountered in elderly women with predilection for the right colon originating in serrated adenomas [24]. In general, they produce poorly differentiated adenocarcinomas with extensive mucin production and are identified as CIMP positive. This CIMP phenotype is associated to recurrence after resection of stage III carcinomas in the proximal colon [25] and conveys a poor prognosis and is associated to resistance to 5-FU-based therapy [26]. There is proposal to classify colorectal adenocarcinomas based on the presence or absence of CIMP [27]. While the CIMP-H tumors are associated with the *BRAF* mutation, the CIMP-L are associated with *KRAS* mutations [27].

• MSI

Millions of mismatch errors occur in the pairing of nucleotides during DNA duplication and cells rely on a robust mismatch repair (MMR) gene system that corrects those mistakes. Base mismatch arises more commonly in short, repetitive areas of the DNA known as microsatellites. The most common gene products responsible for DNA repair are *MSH2*, *MSH6*, *MLH1* and *PMS2* [10,12]. Abnormalities in any of the mentioned proteins that result from mutations or epigenetic hypermethylation (see above) will lead to a microsatellite unstable tumor (MSI-H). High MSI is observed in 15–20% of colorectal carcinomas; most of which are sporadic and only 5% represent cases of Lynch syndrome as described above. Testing for MSI and its role as a biomarker is discussed below.

• Hyperplastic/serrated polyposis syndrome

It has been shown that some hyperplastic and serrated polyps in patients harboring multiple polyps have high levels of MSI (MSI-H) and are associated with loss of expression of the DNA MMR protein MLH1 due to promoter hypermethylation and *BRAF* mutation [28]. These findings may support the concept of a different mechanism for the hyperplastic polyp/serrated adenoma-adenoma-carcinoma sequence in the right colon [29], whereas the traditional serrated adenoma of the left colon is associated with chromosomal instability (CIN) and *KRAS* mutation. The *KRAS* and *BRAF* mutations have been identified in up to 30% and up to 75% of the serrated polyps, respectively [30–32]. The WHO proposed criteria for the clinical diagnosis of the syndrome [33], which predisposes patients to develop CRC in approximately 50% of patients [34].

Lynch syndrome or hereditary nonpolyposis colorectal cancer

This is an autosomal-dominant condition initially described by Whartin in 1913 and then

largely characterized by Lynch in 1966 [35,36]. It is the most common hereditary CRC predisposing syndrome representing about 3–5% of all CRCs. Overall, patients who carry the mutations have a 30–70% lifetime risk of developing CRC, 30–60% risk for endometrial adenocarcinoma and 5–15% for malignancies in other organs including urinary tract, small intestine, ovary, stomach, pancreas, biliary tree and brain. The carcinomas usually arise in small adenomas (5 mm) that show high-grade dysplasia, are located in the right side and show MSI [37]. This MSI is due to defects in the MMR gene, most commonly MSH2 and MLH1. See **Table 1** [37–38].

Molecular biomarkers

Biomarkers in the field of pathology and oncology may allow for the detection of disease, assessment of prognosis or prediction response to certain therapy [39]. The degree of histological differentiation, the depth of invasion in the colonic wall, lymphovascular invasion, the presence of lymph node metastasis or to other body sites have been utilized as part of the TNM system. However, the search for molecular markers for a given patient in order to predict the prognosis and response to therapy advances with the ultimate goal of improving the colorectal cancer patient's life expectancy and quality of life.

MSI testing

Microsatellites are repeats of short nucleotide sequences in the genes prone to frame shifts and base-pair substitutions during replication if DNA MMR are impaired. The deficiency in the MMR system with the subsequent change in the clonality in the repeated nucleotide units is present in tumors due to the inactivation of one of the four MMR genes, namely *MLH1*, *MSH2*, *MSH6* and *PMS2* (postmeiotic segregation increased 2). This MMR protein deficiency in the cancer cells produces the MSI-H phenotype in which the cells may have a germline mutation in those gene proteins or an altered *EPCAM* (*TACSTD 1*) gene [40].

The importance of the diagnosis of Lynch syndrome is based not only on the early prevention of colorectal cancer and other tumors, but also in the therapy modality. Patients with MSI-H present at stage II and III of the disease and have in general a better prognosis. However, they do not respond to conventional alkylating agents (5-FU) and cisplatin [8]. This resistance to 5-FU in stage II tumors appears controversial [5].

There are certain histological features that suggest a high MSI profile including abundant mucin production, poor differentiation, presence of signet-ring cells, lymphocytic infiltration of the tumor, lymphoid aggregates similar to those seen in Crohn's disease and location in the right colon **(Figures 1A & B)** [41]. As such, signet-ring cell and medullary carcinomas belong to this group.

Until recently, the testing was limited to colorectal cancer patients younger than 50 years of age and patients with strong family history of abdomino-pelvic carcinomas. However, now the tendency is to perform universal testing to all colorectal carcinomas in order to find more possible Lynch cases because classical clinical and histopathological features of Lynch syndrome are not present in all patients [42]. Testing for MSI can be done by performing PCR or by immunohistochemical methods to determine the presence or absence of MMR proteins **(Figures 2 & 3 & Box 1)**. MSI testing detects an abnormal number of microsatellite repeats, indicating the CRC arose from cells with defective MMR genes. MMR immunohistochemistry detects the presence or absence of proteins of the MMR genes, suggesting mutation in the gene coding of the protein. Selecting one method over the other depends on their availability and the pathologist's experience [43]. Both methods show similar sensitivity and specificity. The MSI sensitivity ranges from 93 to 97% and its specificity is 83%, whereas immunohistochemistry has a sensitivity of 79–92% with a specificity of 89–100%. In general, immunohistochemistry is carried out initially by the pathologist. A normal expression is identified when any of the tumor

Table 1. Cumulative risk for colorectal carcinomas in mismatch repair mutation carriers by age 70 years.

Mismatch repair mutated gene	Risk of colorectal carcinoma (%)
MLH1	49
MSH2	52
MSH6	12
PMS2	15–20

cells show nuclear immunoreactivity, which can be patchy. In the absence of positive staining in cancer cells one has to observe positive result in the benign cells, which serve as internal controls and they include lymphocytes, stromal cells and non-neoplastic mucosal epithelium.

In most cases, the protein responsible is MLH-1 followed with a significantly lower percentage by MSH-2, MSH-6 and PMS-2. The loss of expression of the MLH-1 protein is frequently associated to sporadic events. However, a minor subset of the patients corresponds to the Lynch syndrome. This distinction can be made by genetic testing, but *BRAF* mutation essentially may rule out familial cancers (see *BRAF* below). On the other hand, patients with loss of expression of the MSH-2, MSH-6 and PMS-2 genes are strongly associated to the syndrome.

The diagnosis of high MSI occurs due to the presence of at least two unstable microsatellites in a panel of five microsatellites (BAT5, BAT26, D5S346, D17S250, D2S123) studied in the clinical practice. A small group of tumors show instability in only one microsatellite and are classified with low levels. Ribic *et al.* found that MSI-H status can be associated with poor response to 5-Fluoruracil-based chemotherapy compared with MSI-L and MSS [8]. This is considered an adverse independent prognostic factor in stage C (equivalent to American Joint Commission on Cancer TNM stage III) colorectal cancers [44]. These cases are also associated to a greater grade of tumoral methylation [28]. Recent studies suggest that another key piece in the diagnosis is the study of adenomas [45–48]. In our hands, adenomas with high MSI associated with carcinoma show the same molecular profile are associated to specific genetic mutations for the Lynch syndrome [49].

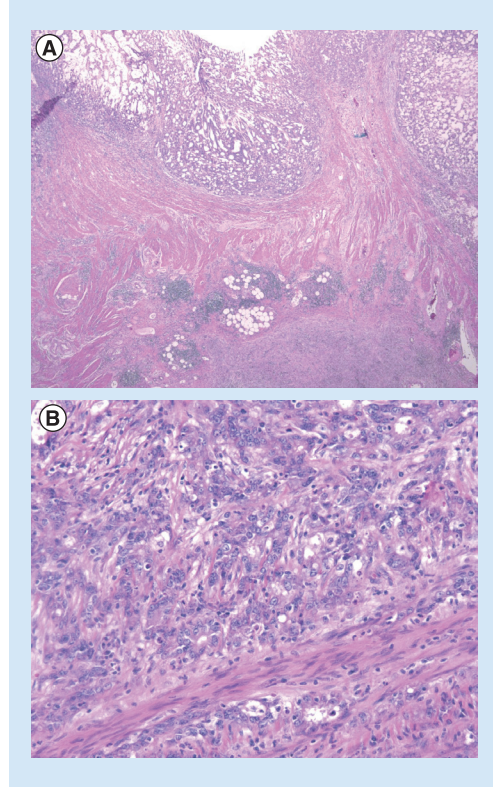

Figure 1. Characteristic features of microsatellite unstable adenocarcinomas. **(A)** Colonic adenocarcinoma showing mural lymphoid aggregates and **(B)** solid pattern of growth with lack of glandular lumen formation, which are features that may be associated to high microsatellite instability.

KRAS as a predictive biomarker

The EGFR also known as Her1/Erb1 is found in the majority of epithelial tissues. It responds to stimuli such as the growth factor among others. The union triggers a cascade of intracellular signals in which *KRAS* is involved that regulates cellular growth, angiogenesis and ability to invade and develop metastatic capabilities by the tumoral cells [50,51].

KRAS is a gene that belongs to the *Ras* family, it is associated to protein-G and is in charge of translating the activation of surface receptors and various intracellular proteins implicated in distinctive steps in the cellular growth [52]. Mutations of the *KRAS* gene result in permanent activation of the intracellular signals in the *RAS*/*MAPK* (*BRAF*) pathway and the *PI3K–AKT* pathway.

KRAS mutations are considered to be an early event and are observed in 20–50% of cases.

Twenty percent of all new cases of colorectal carcinoma present with metastatic disease and 50% will develop metastasis in the course of the disease [53]. Stage 4 patients benefit with the use of anti-EGFR monoclonal antibodies such as cetuximab and panitumumab [54,55] as long as their tumors do not carry *KRAS* gene mutations [56,57]. Because of their high cost and adverse side effects, it is imperative to identify at the beginning those patients that are going to respond to the therapy [58]. Otherwise, only 10% of all colorectal carcinomas will respond to cetuximab. The mutations related to anti-EGFR therapy resistance include those in codon 12 in 80% of cases, in addition to those in codons 13 and 61. However, several studies utilizing *EGFR*

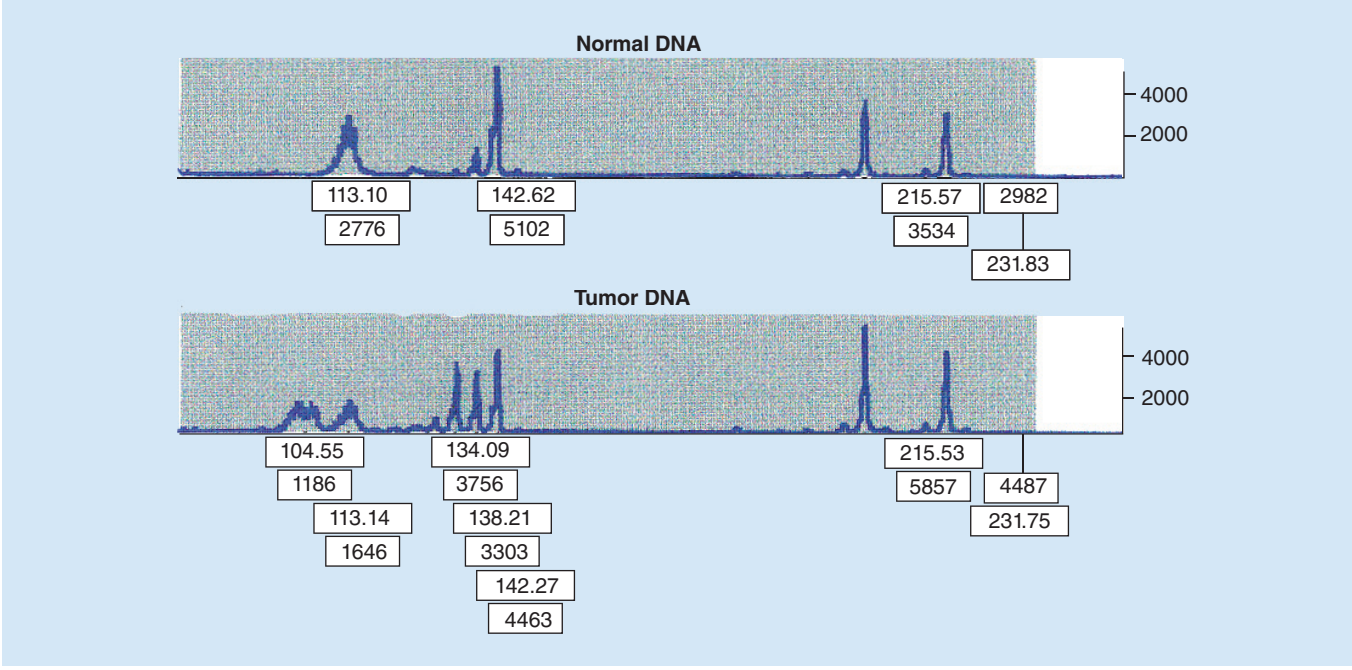

Figure 2. Electropherogram showing the difference in microsatellite expression in normal tissue and tumor.

as a biomarker have not demonstrated that it may be predictive of clinical response.

The detection of these mutations in the *KRAS* gene are indicative that the patients will not benefit from anti-EGFR therapy [52,56] and also predict a shorter disease-free progression [52,55–56]. Currently, 60% of patients with 'wild-type' *KRAS* have a partial or no response with anti-EGFR therapy [55]. This demonstrates the co-existence of other molecular mechanisms involved in the tumoral growth. In fact, there are 'extended *RAS*' mutations (in exons 3 and 4 of *KRAS* and exons 2, 3 and 4 of *NRAS*) in some tumors. These patients may not only benefit from anti-EGFR therapy but may potentially be harmed [58]. Approximately 20% of *KRAS* exon 2 wild-type tumors were found to have one of the extended *RAS* mutations. Extended *RAS* mutations should properly be classified alongside *KRAS* and be considered a contraindication to treatment with anti-EGFR therapy. Altogether, approximately 55% of all metastatic colorectal patients are now considered to be *RAS* mutant, thus negatively impacting the percentage of patients eligible for anti-EGFR therapy, perhaps now with only 40% of patients being eligible. Pathology departments and reference laboratories need to add and update the battery of testing with extended panel. **Box 2** highlights key points for *KRAS* testing, which is currently carried out by performing PCR which is more sensitive than sequencing technique and requires less number of tumoral cells **(Figure 4)**. Different platforms are currently available for molecular studies, including allele-specific PCR, PCR and whole genome or custom panels next-generation sequencing (NGS), posing a challenge due to lack of standardization not only in the analytical-related aspects of the assay but also in the preanalytical (sample-related) and

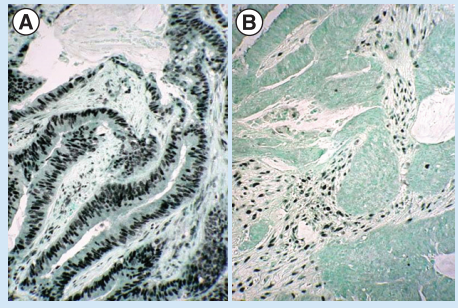

Figure 3. Immunohistochemical staining for (A) MLH1 and (B) MSH2. While the neoplastic cells retain the nuclear staining with MLH1 there is loss of nuclear staining with MSH2. Note the positive nuclear staining in benign stromal cells in **(B)**, this staining pattern is characteristic of Lynch syndrome related to MSH2 mutation.

Box 1. Testing for microsatellite instability.

- Indication: multiple tumors, young patients, co-existence of other cancers and colon cancer, tumors of the right side. Currently, universal testing
- Clinical importance: determines what patients will not benefit from adjuvant therapy with 5-Fluorouracil, predicts a better prognosis and is key to start the search for hereditary cases
- Material tested: primary tumor and/or adenoma by using PCR or immunohistochemistry on paraffin embedded tissue
- Limitations: the sample must contain at least 5% of tumoral cells dependent on the utilized method. The expression of the mismatch repair proteins is variable and may create problems in the interpretation

postanalytical (report-related) aspects of the methodologies.

BRAF & *MLH-1* promoter hypermethylation

BRAF is a member of the *RAF* gene family. It codifies for a serine/treonine kinase and it is activated by its interaction with *RAS-GTP*. The presence of mutations in the *BRAF* gene such as V600E transforms the protein into its active form and thus, determining a constant activation of the *MEK* pathway independent of *KRAS*. Since 60% of patients with wild *KRAS* may not respond to anti-EGFR therapy, it was discovered that those patients carry a mutation for *BRAF*, also responsible for the lack of response and also had shorter survival [59–62].

The *BRAF* gene mutations are encountered in 8–12% of colorectal carcinomas and among these, up to 90% of colorectal carcinomas carry a V600E 'missense' mutation, which implies an exchange of glutamic acid for valin in the codon 600 [63].

This mutation is found in 31–83% of sporadic cancers with MSI-H and it is rare in Lynch syndrome [64,65]. Some authors recommend performing *BRAF* V600E mutational analysis in all cases of tumors with MSI-high associated to absence of MLH-1 and PMS2 [66] before engaging on germline genetic testing for being cost effective [66–68], thus identifying those patients with sporadic tumor. It has been proposed that carcinomas arising from colonic serrated adenomas be tested for *BRAF* mutations, as it has been found that they can be encountered in 74% of serrated polyps and in up to 82% of serrated carcinomas [69]. With rare exceptions, the *KRAS and BRAF* mutations are mutually exclusive. **Box 3** shows key elements of *BRAF*.

CIMP-positive phenotype

Determination of the CpG islands methylation is carried out as part of the various protocols of research and not in the clinical practice. New studies are based on the pyrosequencing as a tool to detect methylated genes [23] and it suggests that the tumors that show this profile would tend to behave more aggressively [25].

PIK3CA mutation

PI3K promote cellular proliferation and survival by inactivating pro-apoptotic proteins such as BAD and Forkhead (FKHR). They can be activated through mutations in *PIK3CA* gene affecting the downstream pathway from *EGFR* and *RAS–RAF–MAPK* pathways. *PIK3CA* mutations are found in 10–20% of colorectal carcinomas and are associated to *KRAS* mutation and MSI. Co-existence of *PIK3CA* mutation in exons 9 and 20 is associated with poor prognosis [70]. However, the regular use of aspirin after diagnosis of colorectal carcinoma has been associated to longer survival [71].

Conclusion & future perspective

The molecular mechanisms described do not explain all the colorectal cancers that occur every year. New research studies are published daily attempting to elucidate the mechanisms involved that bring to field potential biomarkers.

Box 2. Points to remember about extended *RAS*.

- Indication: metastatic disease
- Clinical importance: predicts resistance to anti-EGFR agents and implies shorter disease-free progression
- Material tested: primary or metastatic tumor by a PCR or next-generation sequencing technique using paraffin-embedded tumor tissue
- Limitations: up to 60% of patients do not carry *K-RAS* mutations and will not benefit from anti-EGFR therapy

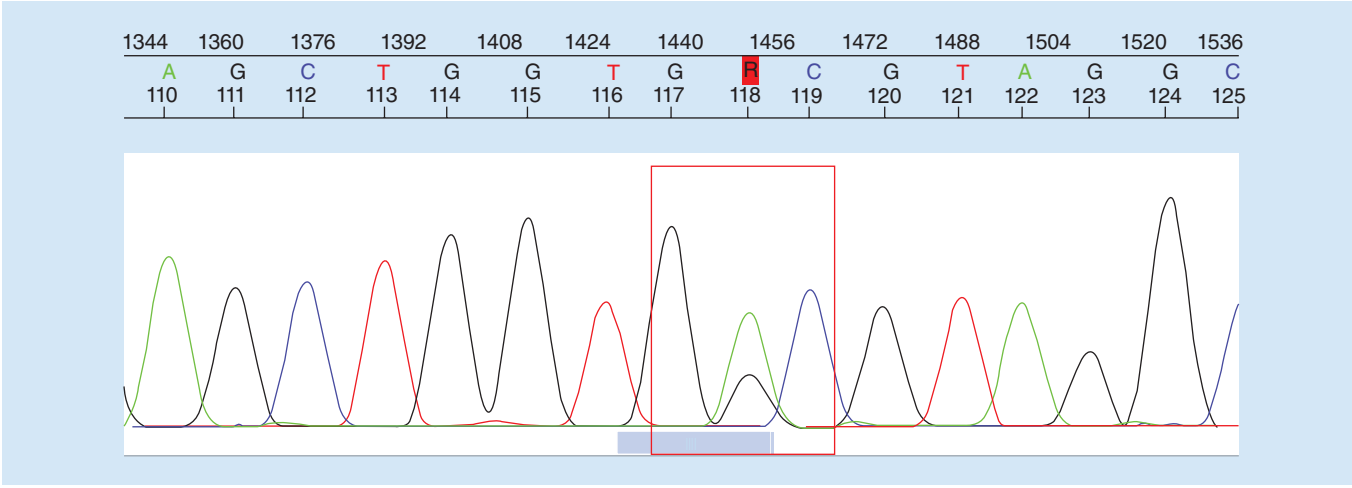

Figure 4. Electropherogram of *K-RAS*. There are allele specific and up to seven mutations that can be determined in the same reaction having the advantage of having a high sensitivity, a few false negatives and it requires less than 5% of tumor in the sample.

One of them is phosphatase and tensin homolog (*PTEN*) expression. *PTEN* is a tumor suppressor gene that inhibits *PIK3CA* signaling. Its immunohistochemical expression is observed in about 75% of cases [72], and it appears to be associated with longer survival in metastatic colorectal carcinoma patients [73,74].

New approaches such as miRNA, the short segments of RNA codifiers have opened horizons in the investigative field. miRNA regulates the post-transcription genetic expression through the cut of the mRNA. The aberrant expression of miRNA is associated to several tumors [75]. Description of several profiles on the expression of miRNA as biomarkers is promising and recently some miRNAs have shown strong association to the development of colorectal cancer and prediction of response to EGFR inhibitors [76,77].

In the near future, most of the institutions will replace the established methodology used in molecular diagnostics for the next-generation sequencing (NGS) method, based on its high sensitivity and the multiplexing options that allows to generate molecular profiles of tumor samples.

Jass proposed a new classification of tumors based on the morphologic and molecular abnormalities that would allow for a better understanding of the tumoral profile and thus, to design specific therapies for each case [78]. This classification includes five groups of tumors, as follows: group 1 (CIMP-H, MSI-H, *BRAF* mutated), group 2 (CIMP-H, MSI-L or MSS, *BRAF* mutated), group 3 (CIMP-L, MSI-L or MSS, *KRAS* mutated), group 4 (CIMP-neg, MSS) and group 5 or Lynch syndrome due to inherited MMR gene mutation (CIMP-neg, MSI-H) **(Table 2)**.

Box 3. Points to remember about *BRAF*.

- Indication:
  - Tumors with high microstatellite instability associated to the loss of MLH-1 protein
  - Metastatic tumors negative for *KRAS* mutation
- Clinical importance:
  - Predicts resistance to anti-EGFR and anti-BRAF agents
  - May separate sporadic colorectal carcinomas from those associated to Lynch syndrome
  - Carries poor prognosis
- Samples to be used:
  - Primary or metastatic tumor utilizing PCR or next-generation sequencing technique on paraffin-embedded tissue with tumor
- Limitations :
  - V600E mutations are currently tested and up to 10% of the *BRAF* mutations are not detected by the techniques utilized today
  - Although the problem of sensitivity can be overcome by tissue microdissection in most cases

Table 2. Proposed molecular classification of colorectal cancer.

Parameter	Group 1 (12%†)	Group 2 (8%†)	Group 3 (20%†)	Group 4 (57%†)	Group 5 (3%†)
MSI	MSI-H	MSI-L/MSS	MSI-L/MSS	MSS	MSI-H
Methylation	+++	+++	++	±	±
APC gene	±	±	+	+++	++
KRAS mutation	-	+	+++	++	++
BRAF mutation	+++	++	-	-	-
TP53	-	+	++	+++	+
Tumor location	Most in the right colon	Most in the right colon	Most in the left colon	Most in the left colon	Most in the right colon
Gender	>Women	>Women	>Men	>Men	>Men
Precursor lesion	Serrated polyp	Serrated polyps	Serrated polyps/ adenomas	Adenomas	Adenomas

†Percentage of colorectal cancers that express that particular genetic pattern.
+: Present in a small percentage of cases; ++: Present in many cases; +++: Present in most cases; -: Never present; ±: May or may not be present; MSI: Microsatellite instability.
Modified with permission from [78] © John Wiley and Sons (2006).

This classification lends support to the view that CRC evolves through multiple pathways.

Further studies in chromosomal losses and the advent of testing in blood and feces for diagnostic biomarkers such as septin 9 (SEPT9) and vimentin (VIM) genes are being performed to determine their applicability in the clinical setting [79].

In the clinical practice, biomarkers are utilized with the objective of deciding the type of therapy, ordering further genetic testing, predict prognosis or simply to prevent the presence of new tumors. The molecular knowledge has allowed a better understanding of the physiopathologic mechanism implicated in the tumoral progression. For instance, alternative mutations in the *KRAS* that may be responsible for resistance to anti-EGFR therapy are being discovered [80]. In the near future, all of this will allow for a classification of the tumor in each patient based on its molecular profile and thus, personalized therapy.

Financial & competing interests disclosure

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Future ONCOLOGY

EDITORIAL

Special Focus Issue: *Future Oncology*: a 10-year anniversary issue

Breast cancer screening: past, present and future

Ismail Jatoi*

"In the future, a less sensitive screening test (and less reliance on technology) might prove to be the best breast cancer screening strategy."

Ironically, improvements in breast cancer therapy and screening technology are very likely diminishing the benefit of mammography screening. Therapeutic advances are reducing both the relative and absolute benefit of mammography screening on breast cancer specific mortality, while advances in imaging technology are increasing the risk of overdiagnosis and hence the risk of a small excess in mortality from unnecessary diagnostic and therapeutic interventions. Alternatives to mammography screening should be considered, particularly in older women, for whom the risk of overdiagnosis is substantial. Screening clinical breast examination (CBE) might be a good alternative, and a randomized trial comparing mammography screening versus screening CBE in older women should be considered as an important step to address the breast cancer screening dilemma. This editorial reflects on the past, present and future of breast cancer screening. Improvements in breast cancer therapy and screening technology are likely reducing the overall benefit of mammography screening, and this editorial discusses this dilemma and the direction that we should take in the future.

Past

In the USA, mammography screening was initiated as a public health strategy around 1980, and has been widely promoted since then [1]. The basis for mammography screening centers on the results of nine randomized controlled trials [2,3]. The first of these trials was the Health Insurance Plan (HIP) trial of New York, initiated in 1963 [4]. In that trial, mammography screening was shown to produce a 30% reduction in breast cancer mortality, but, remarkably, none of the subsequent trials have matched those results [1]. Indeed, the three most recent trials, the Canadian National Breast Screening Study (CNBSS) I, CNBSS II and the UK Age Trial, show no benefit to screening at all [5,6]. This is particularly perplexing when one considers the dramatic improvements in screening technology since initiation of the HIP trial in 1963. The sensitivity of mammography screening has improved over the years, increasing the detection rates of occult cancers. However, breast cancer treatments have improved as well, and adjuvant systemic therapy was freely available to women enrolled in the CNBSS I,

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• therapy

"Improvements in breast cancer therapy and screening technology are likely reducing the overall benefit of mammography screening..."

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CNBSS II and UK Age trials. The failure of these recent trials to demonstrate a mortality benefit from screening might at least partly be due to the availability of effective breast cancer adjuvant systemic therapies for women enrolled in those trials.

Present

In the population, the benefit of mammography screening is likely decreasing, as more effective therapies become available [7,8]. Moreover, improvements in technology have increased the sensitivity of breast cancer screening, thereby increasing the risk of overdiagnosis (i.e., the risk of detecting occult cancers that pose no threat to life and that, in the absence of screening, would never have been detected) [9,10]. This, in turn, has potentially increased the risk of unnecessary diagnostic and therapeutic interventions, and the risk of mortality from those interventions. Thus, with the wider availability of more sensitive screening technology and improved adjuvant systemic therapies for breast cancer, the benefit/risk ratio of mammography screening is likely decreasing.

"...improvements in technology have increased the sensitivity of breast cancer screening, thereby increasing the risk of overdiagnosis..."

The benefit of cancer screening derives from early treatment [7]. If there was no effective treatment for breast cancer, then early detection (and hence early treatment) would offer no benefit. Second, if treatment was equally effective if the cancer was detected earlier or later, then again screening would offer no advantage. Third, as treatments improve, both the absolute and relative benefits of breast cancer screening decrease. To illustrate how improvements in therapy reduce the absolute benefit of screening, consider a cohort of women with clinically detected breast cancer who have, say, a 40% risk of death from breast cancer. Screening advances the time of diagnosis and reduces breast cancer mortality by about 25% (the relative benefit of mammography screening reported in previous trials). In this cohort of women, mammography screening would be expected to reduce the risk of death from breast cancer by 10% (25% of 40% is 10%), and the risk of death from breast cancer would now become 30%. However, if effective adjuvant therapy were available, and if that therapy also has a relative benefit of 25%, and was applied to the cohort of women with a 40% risk of breast cancer death then these women would have a 30% risk of death following adjuvant therapy. For these women, screening would have the same relative benefit (i.e., decreased risk of breast cancer death by 25%), but the absolute benefit would now be only 7.5% (25% of 30% is 7.5%), rather than the previous 10%. This example illustrates how improvements in therapy reduce the absolute benefit of screening.

However, improvements in therapy may also reduce the relative benefit of screening. Consider three groups of patients: those curable if their cancers are detected either clinically or with screening, those curable only if their cancers are detected with screening, and those not curable with either screening or clinical detection. Let us assume that this represents 50, 25 and 25%, respectively, of the total pool of breast cancer patients. These are relative measures (i.e., the percentage of patients relative to the total group of breast cancer patients). As treatments improve, the number of patients in the first group (i.e., those whose cancers are curable with either screen detection or clinical detection) would be expected to increase, while those in the other two groups would decrease. The relative benefit of mammography screening (i.e., those curable only if their cancers are detected with screening) would be expected to decrease with improvements in therapy.

The mammography screening trials measured the effect of screening on breast cancer specific mortality, an ambiguous end point [11]. Clinicians do not always agree on cause of death, and two biases may affect the classification of the cause of death in a screening trial: sticky-diagnosis bias and slippery linkage bias [12,13]. As a result of sticky-diagnosis bias, cause of death is more likely ascribed to a particular cancer if it has been previously diagnosed in a patient. In mammography screening trials, women in the screened group are more likely to be diagnosed with breast cancer, and the sticky-diagnosis bias may therefore result in more breast cancer deaths attributed to women in the screened group. By contrast, slippery linkage bias refers to deaths from diagnostic and therapeutic interventions that are a direct consequence of screening, but not generally attributed to screening. Mammography screening may not only reduce breast cancer specific mortality, but it may impact mortality in other ways as well. For example, abnormal findings on a screening mammogram may lead to interventions to establish a diagnosis (i.e., stereotactic biopsies or perhaps even needle-localized breast biopsies under general anesthesia). These interventions

carry a small potential risk of mortality that is generally not attributed to mortality from breast cancer in a screening trial. Black *et al.* examined the impact of both sticky-diagnosis bias and slippery linkage bias in cancer screening trials, and reported that slippery linkage bias was more likely to influence results of these trials than sticky-diagnosis bias [12]. To account for mortality from diagnostic and therapeutic interventions that result from screening, the preferred end point for any cancer screening trial should be all-cause mortality rather than cancer-specific mortality, but such an end point is not practical because it would require a huge sample size that is not attainable.

Mammography screening results in a substantial rate of overdiagnosis. Indeed, it has recently been estimated that up to 30% of occult malignancies diagnosed with screening mammography would never have been diagnosed in the absence of screening [14]. Large numbers of screened women therefore receive unnecessary diagnostic and therapeutic interventions, and this may result in a small excess in mortality from those interventions. As the sensitivity of mammography screening has increased over the years, the rate of overdiagnosis has increased, and one might speculate that this has produced a small excess in all-cause mortality among women who undergo screening. However, these excess deaths are generally not linked to screening.

Future

Ironically, improvements in breast cancer therapy and screening technology are likely diminishing the benefit of mammography screening. Therapeutic advances are reducing the benefit of mammography screening on breast cancer specific mortality, while advances in imaging technology are increasing the risk of overdiagnosis, and hence the risk of a small excess in mortality from unnecessary diagnostic and therapeutic interventions. Alternatives to mammography screening should therefore be considered. We need to consider the possibility that 'less might be better'. That is, less sensitive screening tests might reduce the risk of overdiagnosis, and hence reduce the risk of mortality from unnecessary diagnostic and therapeutic interventions. Screening clinical breast examination (CBE) should be considered as an alternative to mammography screening, particularly in older women, for whom the risk of overdiagnosis is substantial [15]. In the CNBSS II trial, women were randomized to screening mammography + screening CBE versus screening CBE alone, and there was no difference in mortality between the two arms of the trial [5]. This suggests that, in the era of modern adjuvant systemic therapy, screening CBE might be as effective as screening mammography in reducing breast cancer mortality. A randomized trial comparing screening mammography versus screening CBE in older women should therefore be considered to address this issue. In the future, a less sensitive screening test (and less reliance on technology) might prove to be the best breast cancer screening strategy.

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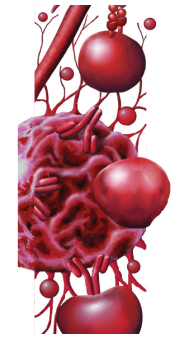
Ask the Experts

Immunotherapy



Immunotherapy for breast cancer: is it feasible?

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Immunotherapy invited leading experts in the field to share their thoughts on two key immunotherapeutic strategies in the field of breast cancer research, vaccines and checkpoint inhibitors.

Interviewed by Ellen Clarke (Commissioning Editor, Future Science Group).

Historically breast cancer has been considered immunologically silent. Patients have had limited access to the types of immunotherapy available to melanoma and lung cancer patients, but this could all be set to change as recent preclinical and clinical studies have highlighted the potential of immunotherapy for breast cancer. Breast cancer is now one of the major cancer types for which new immune-based treatments are being developed.

Leisha A Emens: Department of Oncology, Johns Hopkins University School of Medicine, Kimmel Cancer Center at Johns Hopkins

Q Currently what are the treatment options available to breast cancer patients?

Invasive breast cancer is managed in a multidisciplinary fashion using a combination of surgery, radiation therapy and systemic therapy. Systemic therapies include endocrine therapy, chemotherapy, monoclonal antibody therapy (trastuzumab, perutuzmab, trastuzumab-emtansine (TD-M1) and small molecularly targeted therapies (lapatinib, everolimus and palbociclib). For early stage invasive breast cancer, the goal of therapy is cure. Surgery and radiation therapy are used for local control, where surgical excision removes the bulk of the tumor and radiation is given to treat local microscopic disease that could be left behind. For early stage invasive breast cancer, systemic therapy is used to eradicate disseminated micro-metastatic disease that could ultimately result in relapse and incurable disease. The intensity and type(s) of systemic therapy used with curative intent is matched to the risk of relapse associated with the primary tumor, and to its histologic profile (whether the tumor expresses the estrogen receptor (ER), progesterone receptor (PR) and/or the HER-2). The intensity of systemic therapy is increased in relative proportion to the estimated risk of relapse, with more aggressive chemotherapies generally used for higher risk primary tumors. Chemotherapy is integrated with other systemic therapies based on the histologic profile of the breast tumor. In addition to chemotherapies, endocrine therapy (tamoxifen and/or aromatase inhibitors) is used for early breast cancers that express the ER and/or PR, and HER-2-directed monoclonal antibody therapy (trastuzumab and pertuzumab) for early breast tumors that overexpress HER-2.

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Once distant relapse has occurred, breast cancer is incurable. The primary treatment for unresectable, locally recurrent and frankly metastatic breast cancer is systemic therapy. Because the disease is incurable, the intent of therapy in the metastatic setting is palliative. In this case, therapy is chosen with the goal of both maximizing disease control and minimizing the side effects of therapy so as to maximize quality of life. As with treatment for early stage breast cancer, the choice of systemic therapy is dictated by the biologic profile of the tumor. It is standard to biopsy at first relapse to be sure the biologic profile has not changed, and to tailor therapy to the biologic profile of the relapsed tumor if it has changed. Metastatic breast cancers that express ER and/or PR are managed with endocrine therapies (tamoxifen, aromatase inhibitors, fulvestrant), and metastatic breast cancers that overexpress HER-2 are managed with HER-2-directed therapies (trastuzumab, perutuzmab, trastuzumab-emtansine (TD-M1) and lapatinib). Newer molecularly targeted therapies such as everolimus and palbociclib have shown benefit for metastatic breast tumors that express the ER and/or PR too. Triple negative breast cancer (TNBC) fails to express the ER, PR and HER-2, and there are currently no targeted therapy options available for patients with early or metastatic TNBC in the USA. Chemotherapy remains the only treatment option available for these patients. Surgery and radiation therapy may be used for the palliation of local symptoms.

Q What are the potential advantages of immunotherapy over conventional breast cancer treatments?

Immunotherapy has distinct advantages relative to conventional therapies for breast cancer. One major advantage of immunotherapy is a favorable side effect profile. By contrast, one disadvantage of most conventional breast cancer therapies is lack of specificity, with limiting side effects that result from collateral damage to nonmalignant host tissues. These include nausea and vomiting, hair loss and low blood counts. These side effects are classically associated with most chemotherapy regimens, and neuropathy related to the use of particular cytotoxic drugs poses an added burden for some patients. Additional undesirable side effects of conventional therapy include cardiac dysfunction associated with HER-2-directed therapy, and menopausal symptoms associated with endocrine therapy. By contrast, immunotherapies, particularly vaccines, are generally quite well tolerated. The side effect profile of immune-based cancer therapies, particularly immune checkpoint antagonists, includes autoimmune side effects, which can be serious if not detected and treated early. Importantly, immune-based therapies typically do not cause the types of chemotherapy-related side effects that patients dread (noted above).

A second major advantage of immunotherapy is its ability to circumvent primary or treatment-emergent drug resistance by its unique mechanism of action. Standard cancer therapies frequently fail either due to intrinsic resistance to therapy, or to the emergence of drug-resistant tumor cell clones that result in disease relapse and/or progression. By contrast, cancer immunotherapies are exquisitely specific for and target multiple distinct molecular markers of the tumor, decreasing the likelihood of therapeutic escape and complementing the activities of standard breast cancer therapies. Finally, the greatest advantage of cancer immunotherapies relative to conventional therapies is durability even in the absence of ongoing treatment. A cardinal feature of the immune system is the memory response, where immunity is primed to become activated at the first sign of disease activity. Immunologic memory underlies the durable immune responses observed with immune checkpoint blockade, and the overall survival benefit observed with some vaccine strategies. An established immunologic memory response may obviate the need for continuous therapy as required for conventional systemic therapies. Most importantly, immunologic memory sets the stage for highly effective cancer prevention strategies.

Q Please can you highlight the most promising vaccine candidates for breast cancer, and describe their mechanisms of action?

The most promising vaccine platforms for breast cancer therapy engage the power of dendritic cells to cross prime a coordinated immune response specific for a variety of tumor antigens, and establish a pool of memory T cells for lasting protection from tumor growth. Two strategies come immediately to mind. First, dendritic cells can be isolated from the patient, activated, loaded with tumor-specific antigens and then re-infused into the patient to prime and expand tumor-specific T cells. Second, dendritic cells may be recruited and activated by a vaccine *in situ*. We have used breast tumor cells genetically engineered to secrete the cytokine GM-CSF to cross prime both $CD4^+$ and $CD8^+$ T cells specific for tumor antigens delivered by the vaccinating tumor cells. A combination of dendritic cell-based vaccine or DNA vaccines with GMCSF adjuvant strategies that more effectively engage the innate immune system to optimize immune activation by tumor antigen-specific dendritic cell vaccines are under investigation, with early hints of success in preclinical models and in the clinic.

Q What are the advantages/disadvantages of vaccines over other immunotherapeutic strategies for breast cancer?

Breast tumor vaccines are designed to induce new breast cancer specific T cells, or amplify a pre-existing T-cell response to breast cancer. Other immunotherapeutic strategies-immune checkpoint blockade targeting PD-1, PD-L1, CTLA-4 or TIM-3 among others, low-dose cyclophosphamide or anti-CD25 monoclonal antibodies targeting regulatory T cells, and inhibition of indoleamine 2,3-dioxygenase are designed to alleviate various pathways of immune suppression that keep T-cell responses shut off. Distinct immunotherapeutic strategies – OX-40 and CD137 agonists, for example – help push the T-cell response forward. Still others – VEGF blockade for example – may facilitate T-cell trafficking into the tumor site. These latter immunotherapy strategies require T cells for their therapeutic effect. Tumor vaccines may induce T cells where they did not previously exist, but the efficacy of vaccine-induced T cells may be constrained by active pathways of immune suppression globally or within the tumor microenvironment. An attractive immunotherapy strategy creates and/or amplifies the pool of tumor-specific T cells by effective tumor vaccination or passive transfer (accelerating the immune response), and provides additional signals that promote optimal T-cell activity (co-stimulatory immune modulators or agents that promote T-cell trafficking into the tumor microenvironment) at the tumor site. Regardless, the ultimate goal of cancer immunotherapy is to establish a pool of memory T cells that can control tumor growth and progression in the setting of existing disease, and prevent disease relapse or development in those at high risk of recurrence or first diagnosis of breast cancer. Vaccines have been enormously successful for the prevention of infectious disease. In light of this, their most obvious and powerful role in cancer management may be the prevention of disease in patients at high risk for cancer.

Q Can you highlight any promising results from checkpoint inhibitor trials in breast cancer patients?

Immune checkpoint blockade has been tested in small numbers of breast cancer patients to date. The first report tested tremelimumab, a monoclonal antibody specific for CTLA-4, combined with the aromatase inhibitor exemestane, in patients with advanced ER^+ and/or PR^+ breast cancer. Clinically, the drug combination was relatively well tolerated, and 42% of patients had stable disease for 3 months or more. Treatment was associated with an increase in peripheral $ICOS^+$ T cells, and a significant increase in the $ICOS^+$ effector T cell/$FoxP3^+$ regulatory T-cell ratio. More recently, both the PD-1-specific monoclonal antibody pembrolizumab (MK3475) and the PD-L1-specific monoclonal antibody atezolizumab (MPDL3280A) were reported to have activity in patients with metastatic TNBC. These antibodies have acceptable side effect profiles, and overall response rates of 18–20%. Importantly, the duration of response with PD-1 pathway blockade in TNBC is longer than with standard chemotherapy, with progression-free survival rates at 6 months of 23 and 27% for pembrolizumab and atezolizumab, respectively; some responses were ongoing at the time of report. Both antibodies are now being evaluated in global Phase II and III trials. Notably, a Phase III randomized, double blind trial is testing atezolizumab or placebo in combination with abraxane as first-line therapy for metastatic TNBC. Additionally, a Phase III randomized clinical trial is testing single-agent pembrolizumab versus chemotherapy of physician's choice for metastatic TNBC. Additionally, a Phase II clinical trial is testing single-agent pembrolizumab in distinct populations of metastatic TNBC.

Q What are the limitations of checkpoint inhibitor drugs?

Immune checkpoint blockade is an exciting class of agents for cancer patients, with activity across a broad range of tumor types and durable clinical benefit for most patients who respond. While these immunotherapies transform the cancer experience for those patients who do respond, the fact remains that most patients do not respond. Moreover, the inflammatory side effects associated with these agents can sometimes be life threatening, and must be recognized and managed early in their course. Developing therapeutic strategies that increase the number of patients who can respond to immune checkpoint blockade is critical, and this will be undoubtedly be achieved through combination therapies. Current data suggest that tumors that do not harbor a significant number of T cells or a significant mutational load may be less likely to respond to immune checkpoint blockade, and devising combination strategies to circumvent these limitations to response is critical for increasing the number of patients who can benefit. Furthermore, strategies for dissociating the serious immune-related adverse events associated with immune checkpoint blockade and its antitumor activity will be critical for maximizing the impact of these drugs in the clinic. Finally, at a societal level, the cost of immunotherapy must be considered, and cost–benefit analyses incorporated into late-stage clinical evaluation are essential for determining the proper place of immune checkpoint agents in the management of cancer. It is likely that the unique mecha-

nism of action cancer immunotherapy will render it far more cost-effective than standard cancer therapy.

Q Does immunotherapy have the potential to become a first-line treatment for breast cancer?

Immunotherapy is the next great frontier for breast cancer therapy. It has already shown promise in advanced disease. It is clear that there is place for immunotherapy in the first-line treatment of both metastatic and high-risk early stage breast cancer. One active clinical trial is already evaluating immune checkpoint blockade – atezolizumab, or MPDL3280A – as one component of treatment for metastatic TNBC at first relapse. A major unmet need is for those patients with residual breast cancer after standard neoadjuvant therapy, and immunotherapy is likely to play a major role in the management of these patients.

Q Where do you see the field of breast cancer immunotherapy heading in the next 5 years?

Given emerging data demonstrating the clinical activity of immune checkpoint blockade in metastatic TNBC, immunotherapy has arrived as a potentially viable treatment strategy for breast cancer. It is likely that immune checkpoint blockade will have an important role in the management of locally advanced breast cancer that is treated with systemic neoadjuvant therapy. A variety of other immunotherapies that circumvent immune resistance mechanisms in advanced disease are likely to play a role in the locally advanced and metastatic disease settings. Perhaps the greatest potential for breast cancer immunotherapy lies in decreasing the likelihood of relapse in early stage disease, and in preventing disease in high-risk patients. These advances in immunotherapy truly represent a revolution in cancer therapy, and they will transform the management of breast cancer beyond anything we have seen before. The greatest strength of immunotherapy lies in its ability to prevent disease, and breast cancer prevention is the next great frontier for breast cancer immunotherapy. The next 5 years should see significant efforts applying immune-based strategies for breast cancer prevention.

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Q What are the potential advantages of immunotherapy over conventional treatments?

Surgical intervention has an immediate effect on reducing tumor load. Likewise, chemotherapy and radiation therapy typically induce rapid measurable tumor shrinkage. These interventions may result in enduring beneficial effects in extending overall survival, but all too often the effects of these therapies have limited durability beyond the treatment period. Hormone therapy does not typically induce rapid measureable effects on tumor growth, and the long-term beneficial effects of hormone therapy require extended administration of drugs that block the tumor growth effects of estrogen and progesterone. In all cases, these conventional therapies are accompanied by deleterious side effects that are often debilitating and frequently preclude patient compliance with additional or extended treatments. Immunotherapy has the advantage of inducing long-term immune memory which once established will persist indefinitely to provide a long-lasting immune attack against the tumor. Immunotherapy may require periodic booster vaccinations or intermittent treatments with checkpoint inhibitors for optimized outcomes and may occasionally induce autoimmune sequelae. However, such additional booster interventions and potential autoimmune complications compare favorably to the harsh toxicities and extended side effects of conventional current standards of care and to the morbidity often associated with breast cancer, particularly with the more aggressive forms of breast cancer. The unique elegance of immunotherapy is that once the immune system is effectively induced to attack and destroy the breast tumor, it keeps doing it relentlessly day and night without requiring any frequent, long-term additional interventions. Moreover, this feature of established long-term memory is not restricted to cancer vaccines and appears to occur even following short-term treatment with checkpoint inhibitors that seem capable of inducing effective *de novo* priming to tumor antigens and durable long-lasting immunity with long-term survival.

Q Please can you highlight the most promising vaccine candidates for breast cancer, and describe their mechanisms of action?

There are several proposed vaccines that target the HER2 receptor for EGF known to be overexpressed in a subset of breast tumors. The ongoing Phase III clinical trial headed by Dr Elizabeth Mittendorf at the MD Anderson Cancer Center (TX, USA) involves 11 immunizations with Neuvax™, a DNA vaccine that incorporates an immunogenic HER2 peptide as well as an immune activating cytokine. This trial is designed to prevent recurrence of breast tumors with low-to-intermediate levels of HER2 expression. Another vaccine developed by Dr Brian Czerniecki at the University of Pennsylvania (PA, USA) involves activation of the patient's own autologous dendritic cells (DCs) against peptides derived from HER2. This approach has shown promise in preventing invasion of ductal carcinoma *in situ* (DCIS), a transformed precancerous breast lesion widely believed to be the precursor of many cases of invasive ductal carcinoma. Dr Czerniecki's DC vaccine appears to be selective against estrogen receptor negative/HER2-positive breast tumors. Dr Mary Disis at the University of Washington (WA, USA) has clinical trials in DNA vaccines derived not only from HER2 but also from other self-proteins overexpressed in DCIS and invasive breast cancer including IGFBP2, IGF1R, and a series of breast cancer stem cell antigens. Dr Olivera Finn at the University of Pittsburgh School of Medicine (PA, USA) has long proposed that immunity targeted against MUC1 may provide safe and effective protection and therapy against breast cancer because the heavy native glycosylation of MUC1 presumably protects normal tissues from immune attack whereas the deficient glycosylation of MUC1 in tumors precludes this protection. Dr Joseph Baar at Case Western Reserve School of Medicine in collaboration with Dr Walter Storkus at the University of Pittsburgh School of Medicine have recently initiated vaccination of patients with metastatic TNBC with a DC vaccine that targets peptides derived from tumor-associated blood vessel proteins including among several others, the notch antagonist DLK1. This unique approach is designed to induce a T-cell-mediated response in the tumor microenvironment that compromises the blood supply to the tumor rather than targeting any direct immunity against the tumor itself. Drs Edith Perez and Keith Knutson at the Mayo Clinic (MN, USA) have recently proposed that vaccination against FOLR1 may be effective in preventing recurrence of TNBC, and investigators at the Roswell Park Cancer Center have proposed that vaccination against the cancer testis antigens MAGE-A and NY-ESO-1 may also be effective in treating TNBC. Dr William Gillanders at Washington University has proposed that DNA vaccination against mammaglobin-A (SCGB2A2; secretoglobin, family 2A, member 2) may be effective in preventing breast cancer recurrence due to its overexpression in many human breast tumors, and Dr Songdong Meng at the Chinese Academy of Sciences (Beijing, China) has proposed that vaccination against the heat shock protein 90 kDa beta, member 1 (gp96 or HSP90B1) extracted from human placentas may be effective against breast cancer by inducing immunity against numerous developmentally related peptides expressed in both placenta and breast tumors. Dr Stephen Johnston at Arizona State University (AZ, USA) has proposed that a finite number of nonself neoantigens resulting from frameshift mutations common to many breast tumors can be targeted in a multivalent prophylactic breast cancer vaccine. Finally, we have proposed that safe and effective prevention and therapy against TNBC may be induced by vaccination against α-lactalbumin (LALBA), a protein overexpressed in the majority of human TNBC tumors but 'retired' from expression with age in normal tissues. Clinical trials for our vaccine are planned to begin by the end of 2015 or early 2016.

Q Which stages of the disease are vaccines most effective for?

In our experience with animal models of breast cancer, we have found that the best clinical outcomes occur when the tumor immunity is established early. Indeed, when the tumor has a substantial head start in growing, the induction of immunity has minimal chance to produce any substantive growth inhibition. One can compare the delayed establishment of tumor immunity to giving a world record holder like Usain Bolt a big head start in a sprint race and expecting to catch him and win the race. It is simply not realistic. The induction of an effective immune response involves clonal expansion of high affinity T cells to frequency levels that can produce a clinically relevant immune response. It also involves production of high titers of high affinity antitumor antibodies that may participate effectively in the ultimate demise of the tumor. The completion of this immune process often requires multiple booster vaccinations and typically takes several months to develop and reach maturity. Thus, there seems to be little sense in waiting until the tumor has taken root and has a complete array of dozens of mutations capable of maintaining the adaptive plasticity and immortality of the tumor even in the presence of a powerful vaccine-induced immunity. Considering these issues, it remains perplexing that we still focus predominantly on using tumor vaccines as therapy when we know that vaccines provide

their best impact when used early and pre-emptively to prevent disease. The current paradigm for controlling breast cancer involves waiting for the tumor to manifest and then initiating an offense in the form of surgery, chemotherapy, radiation therapy, hormone therapy, etc. to prevent progression or recurrence of the tumor. Even though preventing recurrence of the breast tumor is often discussed and referred to in terms of disease prevention, it is clearly a treatment and not designed to provide primary pre-emptive immunity against the emergence and growth of newly forming breast tumors. What is urgently needed for optimized control of breast cancer is primary prevention in the form of a prophylactic vaccine that induces immunity in cancer-free and otherwise healthy women, particularly those at high risk for developing breast cancer including previvors with mutations in their BRCA genes. We have proposed that 'retired' self-proteins no longer expressed in normal tissues with age but expressed in emerging tumors may substitute for unavailable viral targets for vaccination and primary immunoprevention of many adult-onset cancers including breast cancer. Our results from extensive preclinical studies provide a rational basis for inducing safe and effective pre-emptive immunity against the emergence and growth of TNBC, the most lethal form of breast cancer and by far the most common form of this disease occurring in women at high genetic risk with mutations in their BRCA genes.

Q How effective are checkpoint inhibitors for hard to treat TNBC cancer tumors?

Many tumors produce PD-L1 that can bind to PD-1 on tumor infiltrating immune cells and transduce a signal that kills the invading immune cells thereby thwarting an effective antitumor immunity. Results from a recent Phase I trial sponsored by Genentech (CA, USA) and led by Dr Leisha Emens at the Johns Hopkins Kimmel Cancer Center (MD, USA) showed that 19% of patients with metastatic TNBC responded to treatment with MPDL3280A, a humanized monoclonal antibody that binds and blocks PD-L1 and thereby allows the tumor-invading immune cells to respond to the tumor. In this way, the drug acts to prevent the TNBC tumors from inhibiting the immune response against itself thereby allowing the patient's own immune system to mount an effective uninhibited antitumor response that inhibits tumor growth. Remarkably similar results were obtained from another recent Phase I trial led by Dr Rita Nanda at the University of Chicago (IL, USA). In this study, the objective response rate was 18.5% in patients treated with pembrolizumab, a checkpoint inhibitor that blocks PD-1. The results of these two Phase I trials are exciting and show great promise for using checkpoint inhibitors against TNBC. However, it is important to recognize that in both studies, less than 20% of evaluable patients responded to treatment with these different checkpoint inhibitors. This significant but modest response rate clearly indicates that there is much room for improvement. Such improvement may occur in ongoing Phase II trials or in future trials involving complementary combination therapies.

Q Do combinations of immunotherapies warrant investigation?

Checkpoint inhibitors that target the CTLA-4 immune inhibitory pathway (e.g., ipilimumab) appear to have their predominant impact on the priming phase of T-cell activation whereas those acting on the PD-1 inhibitory pathway (e.g., pembrolizumab, nivolumab, pidlizumab, MK–3475) or PD-L1 inhibitory pathway (e.g., BMS–936559, MPDL3280A) appear to have their predominant impact on the activity of T cells that are already primed. Thus, it seems reasonable to consider that inhibition of a single inhibitory T-cell pathway may not be sufficient to establish optimized tumor immunity, and that treatment involving both forms of checkpoint inhibitors may provide both enhanced immune priming against the tumor as well as enhancement of any established tumor immunity already in place. The potential synergy that may occur when both pathways are inhibited simultaneously may allow for effective treatment regimens involving lower doses, shorter time courses and diminished toxicities. Combination therapies may also involve co-treatment with a targeted breast cancer vaccine plus ipilimumab during the priming phase of vaccination. Indeed, such combination therapy has recently shown promise in improving overall survival in prostate cancer patients receiving escalating dose of ipilimumab after vaccination with PROSTVAC, a poxvirus-based vaccine against prostate-specific antigen (PSA). Taken one step further, one could consider combining targeted breast cancer vaccination in combination with ipilimumab during the priming phase followed by treatment with an anti-PD-1 or anti-PD-L1 checkpoint inhibitor during the postpriming effector stage of the immune response. In this way, one could sequentially orchestrate an enhanced response to the vaccine in the first phase of combination therapy with one checkpoint inhibitor followed by the induction of an enhanced response to the tumor with another checkpoint inhibitor during a subsequent second phase of treatment. This aggressive combination therapy may be

particularly useful and effective against tumors like TNBC that appear to be only modestly immunogenic and are known to be aggressive and notoriously resistant to currently available treatments.

Q Where do you see the field of breast cancer immunotherapy heading in the next 5 years?

In the next 5 years, we may likely see approval of the first therapeutic breast cancer vaccine and may also see the results of several clinical trials showing efficacy of checkpoint inhibitors in breast cancer treatment, particularly when used in rational combination therapies with each other and with immunogenic breast cancer vaccines. We are already seeing an increase in the number of clinical trials designed to introduce immunotherapies much earlier in the adjuvant setting as soon as possible after surgical intervention so that the activated immune response has the greatest chance to eliminate any residual tumor cells. We may see dramatic advances in the identification of immunogenic breast tumor specific neoantigens that may be highly specific for each tumor and lead to the development of personalized immunotherapies involving tumor-specific customized vaccines. There may also be a breakthrough in the identification of a group of neoantigens common to many breast tumors that could form the basis for developing a multivalent vaccine for treatment and perhaps prophylaxis against defined subtypes of breast cancer. Over the next 5 years, customized immunotherapies will likely become more prominent and successful including active and DC personalized vaccines targeted against individual breast tumor neoantigens or overexpressed self-proteins as well as passively transferred tumor-specific immunity in the form of genetically modified cloned T cells. Finally, clinical trials designed to test safety and efficacy of primary immunoprevention of breast cancer will likely be initiated with the ultimate goal of providing safe and effective immune protection in otherwise cancer-free women, particularly those women at high genetic or familial risk for developing breast cancer.

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Q What are the limitations associated with standard breast cancer treatment?

There is significant heterogeneity in breast cancer even within the subtypes therefore patients develop resistance to current therapies or to progress through multiple lines of established therapies. Currently, there are no validated biomarkers to accurately predict patients that will not respond to specific therapies. Hormone receptor positive tumours do not respond as well to neoadjuvant chemotherapy as triple negative and HER2-positive tumors and the mechanism of this is incompletely understood. Furthermore, adjuvant endocrine therapy provides prolonged disease-free survival; however there are significant differences in recurrence rate and response to therapy between luminal A and luminal B disease. There remain patients that have hormone-positive recurrence even during adjuvant hormone therapy or develop recurrent disease years after initial diagnosis. In metastatic hormone receptor positive disease, patients have to return to chemotherapy after progressing through endocrine options because the tumor develops endocrine resistance. Resistance is also a major limitation in HER2-positive breast cancer, although disease-free survival is much improved after trastuzumab. Furthermore, the hormone receptor positive and hormone receptor negative HER2-positive disease are different disease entities, may have different levels of immune infiltrate and seem to respond to therapy differently therefore these need to be examined separately. Finally, with the addition of adjuvant HER2 targeted therapies, patients are presenting with increased recurrent disease in the brain because it is a privileged site where the targeted therapy cannot infiltrate, therefore studies to determine how to prevent brain recurrence is needed. The highest need for therapy remains in TNBC where there is no targeted therapy. TNBC has also emerged as having five subtypes all of which have different prognoses and response to therapy. More needs to be understood about how different subtypes of TNBC should be treated. Finally, in metastatic triple negative disease the only therapeutic option currently is cytotoxic chemotherapy. TNBC remains the worst prognosis of all the breast cancer subtypes. In pre-invasive ductal carcinoma *in situ* (DCIS), there is a wide range of disease despite all being currently treated as similar. Low-grade DCIS may be able to be followed by active observation whereas high-grade DCIS has a much higher likeliness to progress to invasive breast cancer but currently there are no biomarkers to identify which of these tumors can be safely observed versus need therapy.

Q What are the challenges of developing a personalized breast cancer vaccine?

Breast cancer is typically less genetically unstable than melanoma and lung cancer which may explain why most breast cancers are not effectively recognized and infiltrated by the immune system. Therefore breast cancer also typically has fewer neoantigens to target for personalized tumor-specific neoantigen vaccines. However, many of the overexpressed proteins in breast cancer are conserved between the different breast cancer subtypes. This suggests that conserved tumor-associated antigens may be developed into vaccines that could broadly treat many breast cancer patients and target all of the breast cancer subtypes.

Q Have there been any significant adverse events reported in response to breast cancer vaccines?

No there have not been any significant grade 3 or 4 adverse events currently reported in breast cancer vaccine clinical trials. The most common side effects have been pain and inflammation at the injection sites, transient flu-like symptoms and self-limiting inflammation symptoms. There have also been transient not clinically significant increases in autoimmune serum markers and cytopenias. Most importantly, the significant nonspecific autoimmune side effects such as nephritis, pulmonitis and endocrinopathies seen in other immune therapies have not been seen with breast cancer vaccine therapies.

Q Are there any potential issues that could prevent patients responding to checkpoint inhibitors?

The two issues with breast cancer patients responding to checkpoint inhibitors is that breast cancer overall does not have high levels of immune infiltrate and the immune infiltrates present are typically immunosuppressive. The most common breast cancer subtype is hormone receptor positive breast cancer and this subtype has the lowest lymphocyte infiltrate with only 7% of tumors with greater than 50% lymphocytic infiltrate. Both overexpression of estrogen and treatment with hormone therapy (tamoxifen and raloxifene) have been shown to stimulate a Th2 immune suppressive immune environment. However, even though hormone-positive tumors have not been shown to have prognostic benefit from immune infiltrate, increased FOXP3 immunosuppressive infiltrate predicts worse survival suggesting that the tumor immune environment is important in hormone-positive disease. For hormone receptor positive disease, immune therapy such as vaccines to improve the tumor immune infiltrate and ensure that the immune infiltrate is immune activating (Th1) may then make checkpoint therapy more effective. Unlike hormone receptor positive disease, HER2-positive disease has more immune infiltrate with 11% with lymphocyte predominant (>50% infiltrate). The role of immune infiltrate in HER2-positive breast cancer remains unclear. There is some evidence that increased immune infiltrate has better prognosis in HER2-positive breast cancer but other studies which show no effect. One possible explanation for this discrepancy is that there is some evidence that hormone receptor negative HER2-positive tumors and hormone receptor positive HER2-positive tumors may have different responses to increased immune infiltrate, with the hormone receptor negative, HER2-positive tumors having better prognosis with increased $CD8^+$ T-cell infiltrate. Finally, for the TNBC which does not have high immune infiltrate, vaccination, adoptive T-cell therapy or other immune therapies which increase immune infiltrate to the tumor may allow for better responses to checkpoint inhibitors.

Q Does immunotherapy have the potential to become a first-line treatment for breast cancer?

Yes, immunotherapy has the potential to be first line in both treatment and prevention for breast cancer. However before this can occur, careful evaluation of the immune environment in specific subtypes need to be performed to best understand how to best use immune therapy. Much as has been found in cytotoxic and targeted therapies, no one immune-based therapy will treat all breast cancer subtypes equally. One exception may be in prevention, where the ideal vaccines would target all of the subtypes to activate the immune system and destroy any developing malignancy. The side effects of checkpoint inhibitors may limit their use in prevention, although whether these can be improved with different dosing and treatment schedule remains to be examined. With established tumors, the existing immune environment and stage of disease may determine which immune therapy is most appropriate. For example, in the population of TNBC which has high immune infiltrate, a checkpoint inhibitor may be the only first-line therapy that may be needed. However, for a hormone-receptor positive breast cancer with no immune infiltrate in the tumor a vaccine may be needed to increase immune infiltrate before checkpoint therapy. Finally, there may be a subset of breast cancers which do not respond to immune therapy and require further targeted or cytotoxic chemotherapies, and biomarkers for this subset should be identified so that immunotherapy is not used frontline.

Q Do combinations of immunotherapies warrant investigation?

Yes, a combination of immunotherapies will be important particularly to treat established breast tumors. Furthermore, the role of the immune system with breast cancer therapy is not limited to immune therapies. For example, trastuzumab and other monoclonal antibodies have been shown to be immune therapies, activating antigen-dependent cellular cytotoxicity through natural killer cell recognition of the Fc receptor of the monoclonal antibody. Trastuzumab also can trigger the adaptive immune response, stimulating an adaptive HER2-specific immune response in a subset of HER2 tumors. Cytotoxic chemotherapy can also increase the inflammatory immune environment of the tumor with the tumor cell destruction increasing immune recognition of the tumor and increasing tumor infiltrating lymphocytes. Doxorubicin has been shown to increase cytotoxic $CD8^+$ T cells in breast tumors and paclitaxel has been shown to decrease $CD4^+$ regulatory T cells. Even zolendronic acid has been shown to change the bone immune environment preventing growth and establishment of breast cancer metastases in the bone. Combination immunotherapy can include vaccines with adoptive T-cell therapy and using vaccines or adoptive T-cell therapy with checkpoint inhibitors. However, combining immune therapy with cytotoxic or targeted therapies may further expand the possibilities of immune-mediated options to improve breast cancer therapies.

Q Where do you see the field of breast cancer immunotherapy heading in the next 5 years?

The field of breast cancer immunotherapy will be expanding into both the prevention and the therapeutic setting in the next 5 years. Since clinically the immune system has emerged as important in breast cancer prognosis and development, now the field has to carefully and systematically evaluate the tumor immune environment of the individual breast cancer subtypes to be able to develop rationally designed clinical trials of combination immune therapies to best treat the individual subtypes. Careful evaluation of the tumor immune infiltrate in the breast tumors with the good response to checkpoint inhibitors as well as the breast tumors that do not respond to checkpoint therapies may demonstrate what components of the immune system are necessary for these therapies to function. The role of checkpoint inhibitors in breast cancer and particularly how it differs depending on breast cancer subtypes also has to be evaluated including evaluation of the patients who have failed checkpoint therapy to determine whether they have low immune infiltrate or increased immunosuppressive immune infiltrate preventing response. For prevention studies, it is important to identify clinically relevant antigen targets, particularly as currently early breast cancer biomarkers remain unknown. Also for prevention it is essential to develop a very safe and immunogenic therapy which will allow for the development of memory and a durable immune response, and ideally which will prevent development of all subtypes of breast cancer. The current advances in breast cancer immunotherapy demonstrate that immunology will have a widespread impact on the future of breast cancer therapy.

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The opinions expressed in this article are those of the authors and do not necessarily reflect the views of Future Medicine Ltd.

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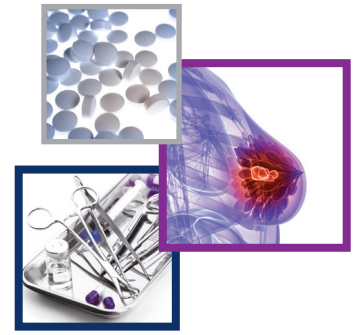
COMMENTARY

Breast Cancer Management



The dawn of genomic medicine: the role of the 100,000 Genomes Project in breast care management

"This project focuses on patients living in England with rare diseases and their families, and patients with cancer."

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On 25 September 2015, University Hospitals of Leicester National Health Service (NHS) Trust recruited its first family to the 100,000 Genomes Project. This project focuses on patients living in England with rare diseases and their families, and patients with cancer. Sequencing 100,000 genomes will enable scientists and doctors to understand more about specific conditions and how we treat them and will greatly enhance the genomic diagnostic capability in the NHS.

Genomics England, a company owned by the Department of Health, is coordinating this work, while 11 NHS Genomic Medicine Centers (GMCs) have been established so far across England. The role of the NHS GMCs is to identify, recruit and consent patients, collect and process samples and capture and submit data through to the validation of the results, to enable clinicians to feed back information to patients and manage the treatment of individuals. University Hospitals of Leicester NHS Trust itself is part of the east of England NHS GMC.

The primary aims of the project are: to bring benefit to NHS patients; to create an ethical and transparent program based on consent; to enable new scientific discovery and medical insights and to aid development of a UK genomics industry. This project has the potential to transform the future of healthcare, find conclusive diagnoses for rare diseases and revolutionize treatments.

This review discusses familial breast cancer susceptibility, the aims, logistics and vision of the 100,000 Genomes Project and how the patients were recruited to the project [1].

Case report

Three sisters were diagnosed with breast cancer within a 15-month period. Their late mother had also been affected. *BRCA1* and *BRCA2* mutation testing had failed to detect a significant alteration and the family were keen to know if the tumors had a strong inherited component to further clarify risks for other family members.

KEYWORDS • *BRCA* • breast cancer • familial• genomics • genomics • personalized medicine • rare disease

"Approximately 20% of these are due to the inheritance of single gene traits such as *BRCA1* and *BRCA2* in an autosomal-dominant manner..."

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The eligibility criteria for familial breast cancer are shown in the Familial Cancer Genome Wheel. The sisters' pedigree **(Figure 1)** shows six females affected with breast cancer over three generations (the three sisters, their mother, a first cousin and maternal great-aunt). The three sisters (indicated by the arrows on **Figure 1**) recruited to the project were diagnosed with invasive forms of breast cancer at an average age of less than 60 years old. The proband, (the first studied affected relative) was diagnosed less than 50 years old. Genetic tests had previously shown that she had not inherited a significant alteration in either the *BRCA1* or *BRCA2* gene.

The three sisters were very keen to participate in the project, which included the completion of consent forms and taking of blood samples. The launch of the project was featured on both the daytime and evening edition of the East Midlands BBC news program, East Midlands Today.

Familial breast cancer

Breast cancer affects approximately one in eight women [2] in the UK. In total, 87% of women with breast cancer have no first-degree relatives with the disease [3]. Approximately 20% [4] of these are due to the inheritance of single gene traits such as *BRCA1* and *BRCA2* in an autosomal-dominant manner (Mendelian trait). Inherited germ line mutations in *BRCA1* and *BRCA2* are identified in approximately 2% [5] of all breast cancer patients overall. Opportunities to identify mutation carriers have historically often been missed [5] which may assist in treatment choices as well as the cascading of risk to other family members.

Familial breast cancer identification

Currently, the NICE guidelines [6] for familial breast cancer management do not recommend prospectively asking about familial breast cancer in primary care. As a result the identification of high-risk relatives is reliant on testing of affected relatives in secondary or tertiary care, or the 'worried well' presenting to their general practitioner with concerns about their family history. It has been previously shown that referrals to clinical genetics departments nationally were doubled directly after the

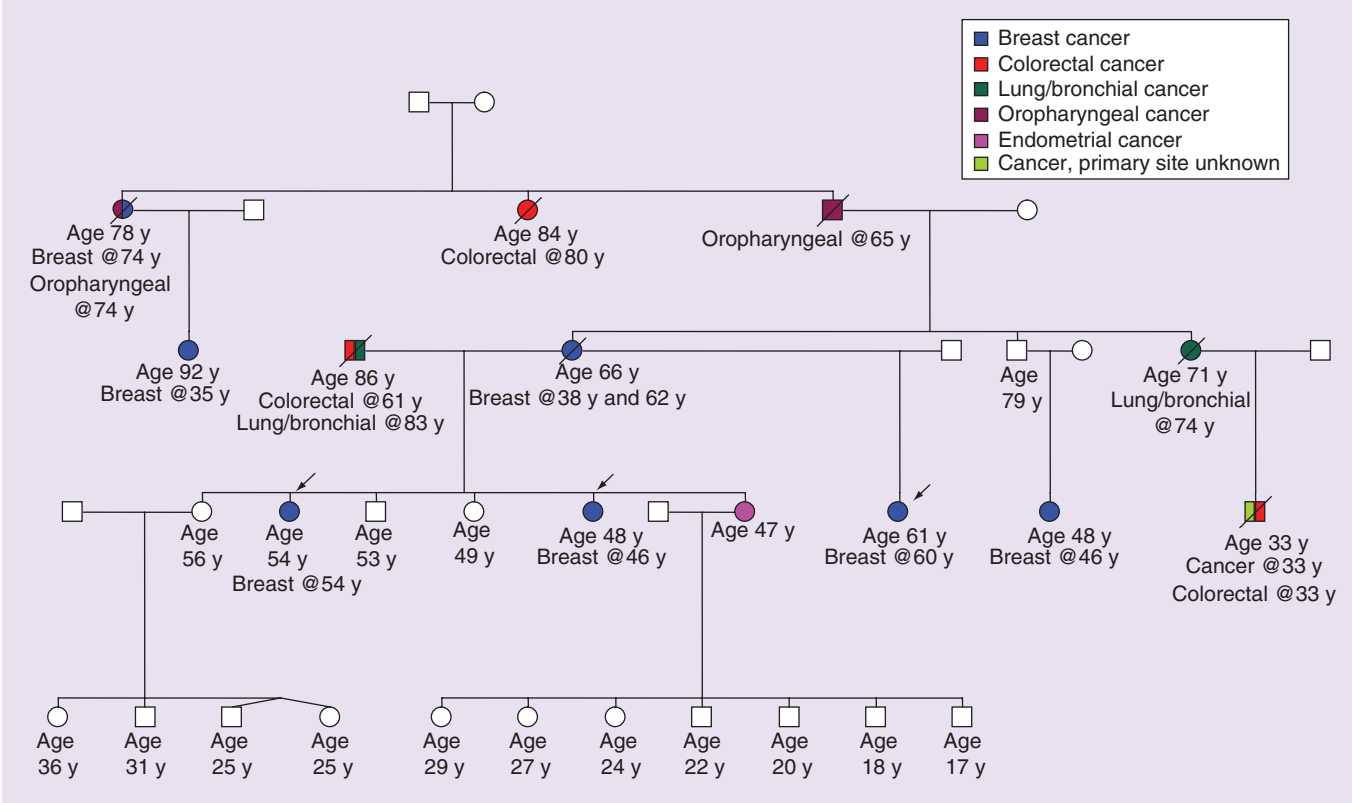

Figure 1. Family pedigree for the first family recruited to the 100,000 Genomes Project at the University Hospitals of Leicester NHS Trust. White squares and circles represent no known health problems. Squares represent males and circles represent females.
Y: Year.

Hollywood actress, Angelina Jolie, admitted in the popular press [7] that she had recently undergone preventative breast surgery as she had inherited a significant alteration in the *BRCA1* gene.

Reasons for identifying mutations

Falling costs and improvements in technology have widened access to *BRCA* mutation testing. This not only helps to clarify risks for relatives but can guide chemotherapy choices and risk of contralateral breast cancer and ovarian cancer susceptibility for the patient and, therefore, eligibility for additional preventative surgery [8,9]. As not all familial breast cancer is due to mutations in *BRCA*, there is great interest in understanding other genetic variation which might be implicated in other families. Future research is also ongoing about the impact of other risk modifiers on *BRCA* mutation carriers and how to use genetic testing on tumor samples and peripheral blood to help predict long-term prognosis and therefore treatment and screening regimens [10]. The introduction of treatments based on the molecular basis of disease has been established for many years, such as the use of tamoxifen in women with estrogen receptor-positive breast cancer and more recently Herceptin® in patients with *HER2* receptor amplification [11]. New technological advances in somatic mutation analysis of tumors and improvements (and reduction in price) to whole-genome analysis has made genomic medicine with more targeted and personalized care based on an individual's mutation load in the NHS a realistic medium-term goal.

The development of the 100,000 Genome Project

With this in mind, the main barriers to wider and more detailed testing and personalized medicine are cost, the re-structuring of clinical genetics laboratories, interpretation of genomic variation, education of the workforce and drug development and availability of modeling. In 2012, the government decided to launch the 100,000 Genomes Project aiming to tackle all of these concerns. The commissioning of a large number of Genomic Medicine Centers as an NHS transformation project is hoped to improve understanding of human variation as well as lower prices and improve technology based on a competitive market model. The alignment of electronic patient records and database phenotypes and facilitating access to pseudonymized data by interested and vetted academic and commercial research groups, provides the best opportunity to help us understand the sequencing results in the short term. The aim is that these records will start to form part of the patient's main clinical record in the longer term and integrate new additional information by connecting with other NHS IT systems such as radiology and pathology. The aim is that these enriched datasets, alongside genomic data, will encourage pharmaceutical industry investment, particularly if international links can be made and a larger patient pool can be identified with rare mutations [1].

What does the project involve for patients?

The project is split between rare diseases where affected patients are recruited (to have their blood taken) alongside their parents to look for inherited and *de novo* traits, and newly diagnosed cancer patients being recruited pre-chemotherapy. Cancer patients have blood and fresh frozen and paraffin-embedded tumor tissue tested to look for germ line and somatic mutations. As well as DNA, samples for potential protein, metabolomics and RNA-based studies are being stored at central depositories, with the DNA being sequenced in Cambridge by Illumina. If mutations are identified, they will need to be validated in an NHS diagnostic laboratory. Patients will be given the opportunity to receive the results of other genes that have been affected that might impact on future health, such as cholesterol, cardiomyopathy or cancer susceptibility. Couples considering having further children will also have the opportunity to receive the results from carrier tests which could result in an autosomal recessive condition, such as cystic fibrosis. The results of the project will not be made available to insurance companies.

Transformation & eligibility

An additional goal of the project is to ensure that patients with a wide number of conditions are able to access this technology to improve the learning for future integration into standard care. Other potentially inherited conditions are included alongside newly diagnosed cancer patients and familial cancer. Examples include familial heart disease **(Supplementary Box 1)**.

Finally, in an attempt to use the project to identify new inherited traits, it has been suggested that only patients who have had prior genetic

"It has been previously shown that referrals to clinical genetics departments nationally were doubled directly after the Hollywood actress, Angelina Jolie, admitted in the popular press that she had recently undergone preventative breast surgery..."

testing where no mutations have been identified but where there is still a chance of a Mendelian mutation being present are being included at this stage, in other words, patients with a significant family history of the same or related condition. We have helped doctors identify potential patients using visual eligibility criteria aids called Genome Eligibility Wheels (see **Figure 2**).

Long-term vision & role of genetics

The vision is that genomic testing of patients with rare diseases at the point of diagnosis by their secondary care team will be the main focus for the molecular identification of inherited disease. Clinical genetics departments will assist alongside bioinformatics teams and academic groups with the interpretation of these traits. Clinical genetics departments will have the additional role of supporting secondary care in counseling patients through the testing process, the cascading of results and guiding the care of patients with identified mutations.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/bmt.15.28

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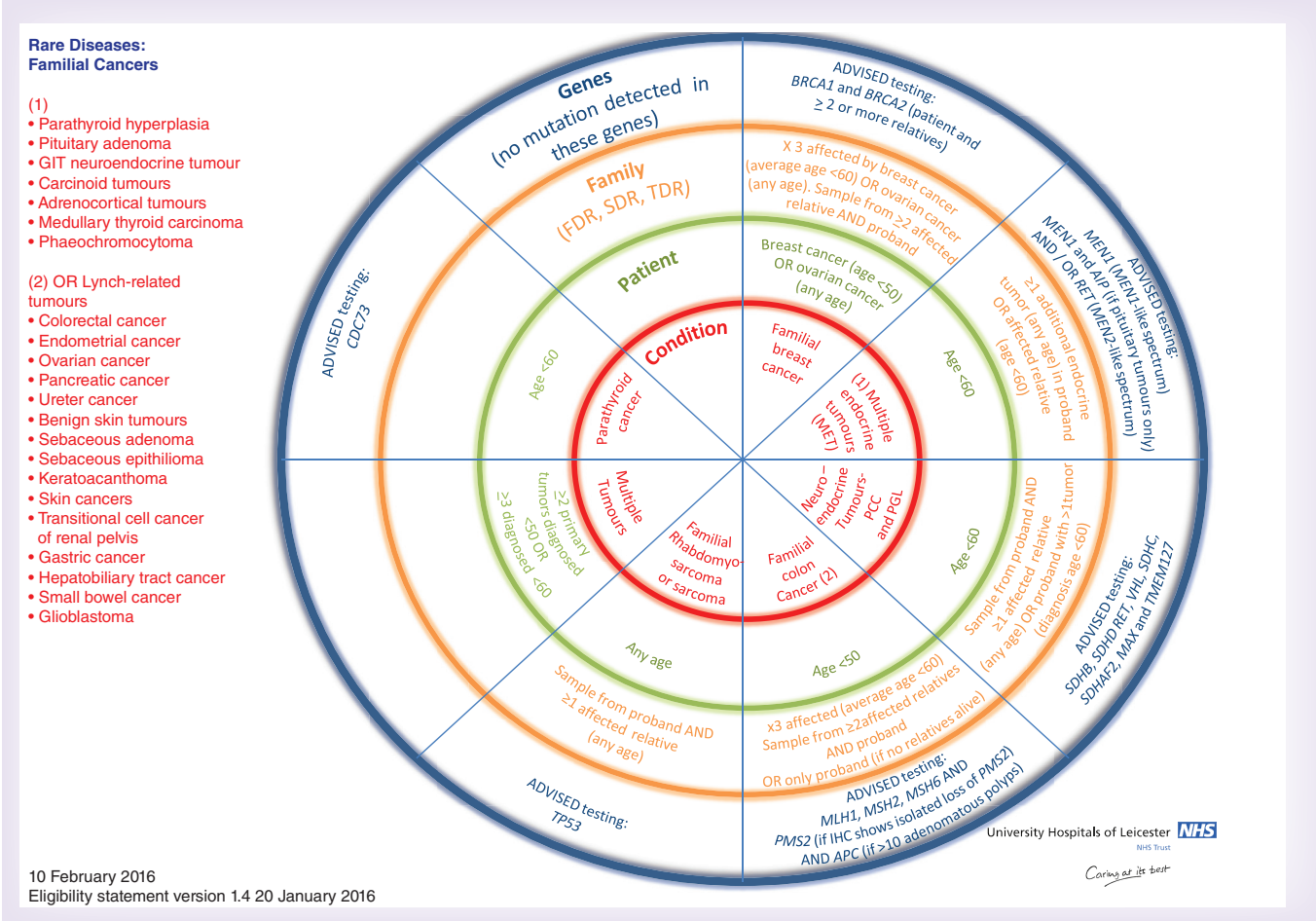

Figure 2. Familial cancer eligibility wheel.
FDR: First-degree relative; MET: Multiple endocrine tumor; PCC: Pheochromocytoma; PGL: Paraganglioma; SDR: Second-degree relative; TDR: Third-degree relative.

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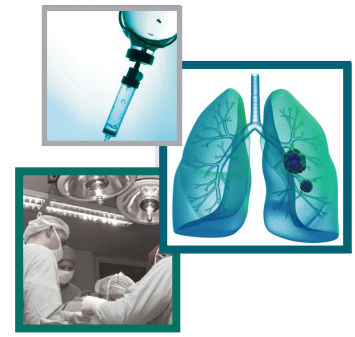

Towards manageable toxicities from targeted lung cancer treatment

Nisha A Mohindra[1,2] & Jyoti D Patel*[1,2]

Practice points

- Targeted therapy is the preferred treatment for patients with *EGFR*-mutant or *ALK*-translocated NSCLC.
- Toxicities associated with targeted therapy are unique and differ from that of cytotoxic chemotherapy.
- While targeted therapy offers increased autonomy and convenience for patients, management of toxicities and compliance becomes more challenging for physicians.
- Early recognition and effective management of toxicities are key to ensure patient compliance and to maintain quality of life of patients treated with such agents.

Targeted agents are now considered standard of care for patients whose tumors possess a sensitizing mutation in *EGFR* or *ALK* rearrangement. As the toxicity profiles of these agents differ significantly from that of cytotoxic chemotherapy, physicians need to be cognizant of the clinically relevant adverse events and manage them aggressively. Early recognition of these toxicities is vital to ensure medication compliance and maintain quality of life for patients. As more novel agents enter the treatment armamentarium, such as third-generation EGFR and ALK inhibitors, it will be important for physicians to understand class-specific toxicities and rare but serious side effects associated with these drugs.

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• afatinib • ceritinib • crizotinib • dermatologic toxicity • diarrhea • erlotinib • NSCLC • targeted therapy

Lung cancer remains the leading cause of cancer-related mortality worldwide [1]. Despite the use of platinum-based chemotherapy, 5-year survival for lung cancer patients remains poor. Efforts to understand the pathogenesis of disease have resulted in the identification of molecular subtypes within NSCLC that have distinct clinical features and oncogenic drivers for tumor growth. Activating mutations in *EGFR* are found in 15% of NSCLC patients in the USA and 2–7% of NSCLC possess rearrangements of the *ALK* gene. Targeted agents are now being utilized in these molecular subsets and have demonstrated superiority over cytotoxic chemotherapy. Further, these agents are also used in the second- and third-line setting in patients whose tumors lack these specific molecular features with limited efficacy [2,3]. Physicians need to be cognizant of the clinically relevant toxicities associated with targeted therapies, as adverse events can lead to medication noncompliance and reduced quality of life for patients. This article will provide an overview of the toxicities associated with

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US FDA-approved targeted therapies (excluding those currently in clinical investigation, monoclonal antibodies and immunotherapy) and suggestions for management.

EGFR tyrosine kinase inhibitors

The EGFR has been an attractive target in NSCLC for over a decade. EGFR, also known as HER1 or ErbB1, is a member of the ErbB family of receptor tyrosine kinases [4]. Once activated, downstream signaling leads to activation of key intracellular pathways involved in proliferation, survival, angiogenesis and metastatic spread [5]. There is now plentiful evidence that in patients whose tumors possess sensitizing mutations in the *EGFR* gene (exon 19 deletions, L858R point mutation in exon 21), use of an EGFR tyrosine kinase inhibitor (TKI; gefitinib, erlotinib, afatinib) is superior to chemotherapy in terms of overall response rate and progression-free survival [6–15].

Gefitinib, erlotinib and icotinib are reversible inhibitors of the EGFR tyrosine kinase, whereas afatinib is an irreversible pan-ErbB inhibitor. Icotinib is not FDA approved in the USA; however, erlotinib, gefitinib, and afatinib are currently utilized. Two of the most common side effects associated with these EFGR TKIs (gefitinib, erlotinib, afatinib) are rash and diarrhea, and while these events rarely result in permanent treatment discontinuation, they can affect a large number of the patients. With afatinib, for example, rash and diarrhea caused treatment termination in <2% of patients among the Phase III trials; however, almost 90% of patients experienced some grade of diarrhea during treatment. Further, 30–50% of patients required dose reductions of afatinib during Phase III trials, emphasizing the importance for physicians to understand drug-specific toxicities and proper management of these agents [6,7].

• Papulopustular skin rash

A rash can occur in 50–80% of patients treated with EGFR inhibitors. [6–17] The rash is typically characterized by inflammatory papules and pustules. Various descriptions have been used to describe these skin changes such as folliculitis, acneiform, acne-like and monomorphous erythematous maculopapular changes to name a few [16,18–20]. However, it is important to note that while the rash does resemble acne vulgaris, the skin reaction is not associated with comedones and is pathologically distinct from acne vulgaris [21]. Biopsy and cultures from these skin changes revealed that it is largely an inflammatory process [21]. Recommended terms to describe these skin changes include pustular/papular/follicular rash or eruption [16].

Common risk factors for development of rash include age (age >70 years), gender, smoking status (nonsmoker > smoker), skin phototype and UV exposure [22–25]. The pathophysiology of these skin changes is complex, and results in part, from loss of normal EGFR signaling in cells that depends on this pathway and a rise in inflammation [4,26–30]. The most frequently affected areas are the face, V areas of the upper chest/back and less commonly the extremities, abdomen or scalp. Skin eruptions usually arise within the first 2 weeks of treatment and a distinct progression of clinical symptomology has been described [31,32]. The first signs of skin changes occur within the first few days of therapy and involve sensory changes, erythema or edema. During weeks 1–3 of therapy, patients may develop erythematous papules or pustules, followed by crusting in weeks 3–5, ending with erythema, telengiectasias and xerosis (skin dryness) in weeks 4–6. Even with resolution of the rash, erythema and xerosis can persist for a much longer time frame (months to years). Despite continued use of the agent, most patients experience an improvement in their symptoms over time. The rash is reversible and will resolve after discontinuation of the agent, usually within 4 weeks; however, some patients are left with persistent hyperpigmentation [18]. Other late but notable dermatologic reactions from these agents include painful fissures on the fingertips, palms and soles, hair changes (hirsutism and eyelash curling) and paronychial inflammation in the nail folds of the fingers and toes which typically occur months after use of these agents [18–19,33].

Most rashes are grade 1 or 2 (according to National Cancer Institute, Common Terminology for Adverse Events) and can be managed effectively without disruption of treatment. However, for grade 3 or higher, skin changes that do not respond to supportive measures, dose reduction and/or brief or permanent cessation of therapy may be necessary. There have been randomized controlled trials evaluating prophylactic measures to reduce or prevent skin changes that will be discussed below. However, there are a lack of evidence-based data evaluating 'reactive' interventions directed at these symptoms once they have

occurred. Most recommendations for reactive treatments come from anecdotal experience, practical guidelines or expert panels [16–17,20,22,27,34–38]. Treatment of these skin changes vary by country and there are several large national panels available for clinician use [35,37,39]. Some recommended prophylactic measures for anti-EGFR-related skin changes are discussed below.

• Prophylactic measures

There have been a number of trials evaluating the role and efficacy of prophylactic skin-directed measures when using EGFR-directed therapy and suggest that prophylactic treatment seems to benefit patients [40–44]. The Pan Canadian Rash Trial with EGFR Inhibitors was a Phase III trial that assessed the optimal management of rash secondary to erlotinib [43]. In this study, 150 patients were randomized into three interventional arms prior to the start of therapy (arm 1 = prophylactic treatment with minocycline 150 mg twice daily (b.i.d.) on day 1 of erlotinib, arm 2 = topical clindamycin and hydrocortisone with or without minocycline upon rash occurrence depending on grade, or arm 3 = observation unless rash severe). Prophylactic minocycline reduced the occurrence and severity of rash and allowed patients to remain on treatment 50% longer than the other 2 arms. Similar results had been seen with prophylactic skin therapy in the STEPP trial for patients with colorectal cancer [42]. Preemptive treatment with skin moisturizers, sunscreen, topical steroid and doxycycline reduced the incidence of grade 2 or higher skin toxicities by 50% and patients reported less impairment in quality of life (QOL). Prophylactic use of oral tetracyclines has also reduced the incidence and severity of rash associated with afatinib [44]. In an open labeled, randomized, controlled trial, 90 patients receiving afatinib for advanced NSCLC were randomized to preemptive tetracycline 250 mg b.i.d. for 4 weeks or to reactive treatment. Tetracycline use reduced both the incidence of rash as well as any rash greater than grade 2 in severity without impact on response rate, progression-free or overall survival. Tetracycline use had no effect on reducing the incidence of other dermatologic toxicities such as paronychia, xerosis, mucositis, folliculitis and skin fissures.

However, not all prophylactic measures have resulted in clinical benefit and some may actually aggravate skin-related toxicities. Prophylactic use of topical retinoids is not recommended as it can cause significant skin irritation [41]. In a randomized Phase II trial, patients were randomized to 4 weeks of oral minocycline versus placebo. All patients applied 0.05% tazarotene, a topical retinoid cream, to both sides of their face. Prophylactic minocycline resulted in decreased severity of rash in the first month of therapy; however, tazarotene made no difference and actually caused increased skin irritation.

Thus, prophylactic oral tetracyclines with topical corticosteroids should be considered for patients initiating EGFR inhibitors. Preemptive treatment should continue for the first 4–6 weeks of treatment [22,36]. Either oral doxycycline 100 mg b.i.d. or minocylcine 100 mg b.i.d. can be utilized, choice of which agent can be based on side effect profile of each drug. Patients should also apply a topical corticosteroid such as hydrocortisone cream 1% to affected areas b.i.d.. Patients should be educated that they can use makeup to camouflage skin changes without exacerbating symptoms. Summary of prophylactic measures found in **Box 1**.

• Diarrhea

Diarrhea is another common side effect of EGFR TKIs [6–14]. The exact mechanism underlying the diarrhea is unclear but it is thought to be secretory in nature resulting from excess chloride secretion [45]. Symptoms typically begin within the first 4 weeks of starting therapy but can arise even after the first few doses. [46,47]. At the first onset of symptoms, patients should be encouraged to eat a bland diet and use antidiarrheal agents such as loperamide. A common algorithm for loperamide use is to take two 2 mg loperamide tablets after the first episode of diarrhea, followed by one 2 mg tablet after every loose bowel movement up to the maximum allowed daily dose of 20 mg/day [20]. Patients

Box 1. Prophylactic measures for EGFR inhibitor-associated papulopustular skin rash (to be done anywhere from 2 to 6 weeks).

Topical measures

- Skin moisturizers applied twice a day
- Sunscreen applied twice a day
- Hydrocortisone 1% cream twice a day
- Clindamycin 1%

Systemic measures

- Minocycline 100 mg twice a day (can be less photosensitizing)
- Doxycycline 100 mg twice a day (can be used in patients with renal dysfunction)
- Tetracycline 250 mg twice a day

should continue loperamide until bowel symptoms stop for at least 12 h. If grade 2 diarrhea continues for >2 days, the patient should be evaluated for signs of dehydration. Dose interruption is recommended until diarrhea improves to grade 1 or less (Box 2) [47]. If the diarrhea fails to improve with this regimen, an infectious etiology should be ruled out as well. Fluid and electrolyte balance are important during this time frame and patients should be encouraged to drink 3 l of fluids, including liquids that have electrolytes and glucose. Spicy or fried foods and dairy products exacerbate bowel symptoms and should be avoided until symptoms improve.

ALK inhibitors

• Crizotinib

There are now two FDA approved agents for the treatment of *EML4–ALK*-positive NSCLC, crizotinib and ceritinib. The *EML4–ALK* fusion oncogene arises from inversion on chromosome 2 that joins EML4 to ALK; the resultant fusion gene causes ligand-independent activation of ALK [48]. Crizotinib, a dual ALK and c-MET inhibitor, has shown impressive response rates and improved progression free survival in *ALK*-positive NSCLC as both first-line therapy and after progression on platinum-based chemotherapy [49–51].

The most common adverse events with crizotinib are visual changes, diarrhea, nausea, edema, fatigue, neutropenia, rash and elevated transaminases [49–52]. Other less common but clinically relevant adverse events (AEs) include bradycardia, hypogonadism in men and decreased creatinine clearance. The most serious adverse events with crizotinib were hepatic events and pneumonitis. Grade 3 or 4 hepatic events occurred in 14–16% of patients in randomized Phase III trials of crizotinib and were managed with dose reduction or treatment delays. Pneumonitis occurred in 1–2% of patients and resulted in treatment discontinuation. Although the incidence of adverse events of any cause was higher with crizotinib versus chemotherapy, patient reported outcomes were more favorable with crizotinib therapy [49,50]. When compared with first- and second-line chemotherapy, the rate of treatment-related adverse events leading to permanent discontinuation of treatment were similar between crizotinib and chemotherapy, 5 versus 8% in the first-line setting and 6 versus 10% in the second-line setting, respectively [49,50]. However, duration of therapy with crizotinib was much longer than chemotherapy in both settings.

Gastrointestinal side effects occur within 2–5 days of starting therapy with crizotinib and are managed in a similar fashion to those mentioned in the section on EGFR TKI-related diarrhea [53]. We will describe some of the other unique side effects of crizotinib and their management below (Box 3).

Box 2. Considerations for therapy-associated diarrhea.

- At first onset of diarrhea, take two 2 mg loperamide tablets, followed by one 2 mg tablet after every loose bowel movement up to the maximum allowed daily dose of 20 mg/day
- Continue loperamide until bowel symptoms stop for at least 12 h
- If grade 2 diarrhea continues for >2 days, the patient should be evaluated for signs of dehydration

• Visual changes

Visual changes are the most common side effects with crizotinib and have been reported in >60% of patients in Phase II trials of crizotinib [49,50]. Visual disturbances usually begin after 2 weeks of therapy, tend to be low grade (1 or 2) and do not typically require dose adjustment. A Visual Symptom Assessment Questionnaire was done on patients enrolled in the Phase II trial of crizotinib (PROFILE 1005) on day 1 of each treatment cycle to better characterize and assess the impact of these visual changes [54]. In this study, patients most often described the visual changes as flashing lights, floaters, overlapping shadows, difficulty adapting to lights and seeing at night. Patients reported that most events lasted <1 min in duration and occurred less than every day. Majority of patients reported that these effects had no or minimal impact on activities of daily living. Almost 200 patients (21–27% of patients) from PROFILE 1005 underwent ophthalmologic assessments, which revealed no objective abnormalities attributable to crizotinib [55]. There has been a case report of optic neuritis and blindness in a patient receiving crizotinib; however, other factors such as radiation may have contributed to these vision changes as well [56]. Thus, baseline or routine ophthalmologic assessment is not needed during crizotinib therapy unless symptoms are significant (such as photopsia or floaters) or worsen over time [57]. There are little data on whether visual disturbances are significant enough to impact the ability to drive [53].

• Bradycardia

Crizotinib will cause grade 1 and 2 bradycardia in 5% of patients [57]. The exact mechanism for bradycardia is unclear; however, some suggest

Box 3. Considerations for crizotinib-related side effects.

Visual changes

- Observation unless significant changes presents (floaters, photopsia)

Bradycardia

- Stop concomitant medications (β-blockers, calcium channel blockers)
- Correct electrolyte disturbances (sodium, potassium, calcium)
- Consider brief cessation of medication if heart rate <60 beats per min

Hypogonadism

- Check baseline testosterone levels in males at initiation of therapy
- Periodically check testosterone levels throughout treatment course and at onset of symptoms such as fatigue, loss of libido or changes in mood

it may be due to MET inhibition as similar effects are seen with other MET inhibitors [58]. Pharmacokinetic and pharmacodynamic studies from the Phase I dose-escalation study of crizotinib revealed an average decrease in heart rate of 2.5 beats per min (bpm) with every 100 ng/ml increase of serum crizotinib concentration [59]. There was also a 2.9 msec increase in Qtc (QT interval corrected) seen during peak concentration with the 250 mg b.i.d. dosing. In a single institution, retrospective study of patients enrolled in the PROFILE 1001 and 1005 trials, majority of patients (90%) had at least one episode where their heart rate decreased by at least 10 bpm from baseline [58]. The average decrease in heart rate in this cohort was 26 bpm, but notably, most patients remained asymptomatic and had no associated QT prolongation. Further exploratory analysis from this study noted a correlation between sinus bradycardia and clinical response rate; however, prospective analysis is warranted to understand this phenomenon better.

Recommendations for managing bradycardia include assessment of the patient's cardiac history, concomitant medications (β-blockers, calcium channel blockers or other medications that may affect cardiac conduction) and presence of symptoms. We commonly will stop treatment if heart rate <60 bmp and consider reintroduction of therapy once heart rate returns to 60 bpm or greater. One could consider continuing therapy despite bradycardia for patients with no prior cardiac history, who remain asymptomatic and who are deriving clinical benefit from the agent. There have been case reports of asymptomatic bradycardia with heart rates ≤45 bmp where treatment was continued without development of symptoms; however, patients were carefully educated on symptoms of bradycardia and to seek immediate medical attention at onset of such symptoms [60]. It is important to correct all electrolyte abnormalities and assess electrocardiograms in patients with bradycardia to assess for Qtc prolongation.

• Peripheral edema

Peripheral edema is a late adverse event of crizotinib, with median onset at 85 days [53]. It is thought to be from cumulative exposure to the agent and rarely resolves on its own. Compression stockings, a low sodium diet or diuretics can be utilized as needed.

• Hypogonadism

A rapid decrease in total and free testosterone levels can be seen within weeks of starting therapy with crizotinib [61,62]. Low testosterone levels can be seen in patients with advanced malignancy; however, a study found that 84% of patients treated with crizotinib experienced low-testosterone level when compared with 32% of non-crizotinib-treated advanced NSCLC patients [62,63]. Testosterone levels should return to normal with cessation of treatment. The exact mechanism underlying this phenomenon is unclear but appears to be due to a central effect (hypothalamic–pituitary axis) as there is presence of concurrent declines in follicle-stimulating hormone and luteinizing hormone [62]. It has been recommended that men treated with crizotinib have baseline testosterone levels drawn prior to start therapy and have levels rechecked periodically and at first signs of hypogondism [64]. It is important that physicians be aware of this side effect and monitor specifically for symptoms of hypogonadism. If low testosterone levels are detected, even in the absence of symptoms, patients should be referred to endocrinology to discuss whether testosterone replacement is appropriate [62]. Hormone replacement has been shown to benefit a small cohort of symptomatic patients; however, larger studies are needed [62].

• Decreased creatinine clearance

There is now evidence that crizotinib may cause a decrease in estimated glomerular filtration rate (eGFR) [65]. A retrospective analysis of patients on crizotinib noted that eGFR declined over the first 12 weeks of treatment; however, greatest decrease was seen in the first 2 weeks. In over half of patients (56%), eGFR returned to baseline once therapy stopped. For the remainder of patients, eGFR returned to 84–97% of baseline. Although the cause is not certain, it is thought that the effect on eGFR is due to drug-induced changes in tubular creatinine secretion rather than a direct nephrotoxic effect [65].

• Ceritinib

Ceritinib is a second-generation TKI of ALK that is 20-times more potent than crizotinib and had preclinical activity against both crizotinib naive and crizotinib-resistant xenograft models [66–68]. Phase I data demonstrated that ceritinib had substantial antitumor activity in both crizotinib naive and crizotinib pretreated patients with *ALK*-rearranged NSCLC leading to its FDA approval for patients intolerant of or who had progressed on crizotinib [69]. Furthermore, ceritinib has demonstrated activity against brain metastases, even in patients with no prior brain radiotherapy [70,71].

The combined results from the dose-escalation and expansion cohorts of the Phase I trial revealed the most common adverse events were nausea, diarrhea, vomiting, fatigue and elevated alanine aminotransferase levels. The most common grade 3 or 4 toxicities were elevations in liver function tests (aspartate aminotransferase [AST], alanine aminotransferase [ALT]), diarrhea and elevated lipase levels. At the maximum-tolerated dose of 750 mg, more than half of the patients (62%) required a dose reduction. Updated results from the expansion cohort patients were presented at the annual American Society of Clinical Oncology meeting in 2014 [70]. Overall, 52.2% of patients required a dose reduction and 9.4% of patients discontinued ceritinib due to an adverse event. In this updated report, the most common AEs were diarrhea, nausea, vomiting, fatigue and elevated ALT. The most common grade 3 or 4 AEs were elevated AST and ALT.

Given the high rate of dose reductions seen on study, it is important to evaluate patients starting on ceritinib frequently for toxicity and to monitor liver function tests carefully. We recommend that patients initiating therapy with ceritinib be seen on a weekly basis at first and that liver function tests be evaluated at least every 2 weeks for the first 2 months.

Conclusion & future perspective

Targeted therapy not only provides clinical benefit and improved quality of life for select NSCLC patients but also offers a shifting paradigm for how we treat patients. Oral antineoplastic agents offer patients increased convenience and autonomy, but adherence and safety monitoring become a real concern for physicians and patients. There is evidence that in the palliative setting, patients prefer oral chemotherapy rather than intravenous therapy as it is more convenient, patients can receive treatment at home and there are less issues with venous access [72,73]. However, ensuring patient compliance is key, as noncompliance not only negatively impacts clinical outcomes and efficacy but also can increase healthcare costs [74,75]. Additionally, factors such as monitoring drug–drug interactions and ensuring proper patient education on how to take these drugs are factors to consider when using targeted agents. Early and frequent toxicity assessments as well as measures that reduce adverse events are important factors to ensure patient adherence.

As more promising agents enter the treatment arena, these factors will become ever more important. Currently, there are promising targeted agents in late phase clinical investigation for patients with *EGFR*-mutant and *ALK*-rearranged tumors. Rociletinib (CO 1686) and AZD9291, both third-generation EGFR TKIs, have demonstrated promising activity in patients whose tumors harbor sensitizing *EGFR* mutations as well as the acquired resistance mutation T790M [76,77]. These agents are thought to have less effect on wild-type *EGFR*, which reduces some of the toxicities seen with first- and second-generation EGFR TKIs. However, these agents have unique side effects as well, such as hyperglycemia seen with rociletinib [77,78]. Similarly, alectinib, a third-generation ALK inhibitor, has demonstrated clinical efficacy in *ALK*-rearranged NSCLC after progression on crizotinib and has promising activity within brain metastases [79,80]. As more novel agents enter the treatment armamentarium, it will be important for physicians to understand class-specific toxicities and rare but serious side effects associated with these agents.

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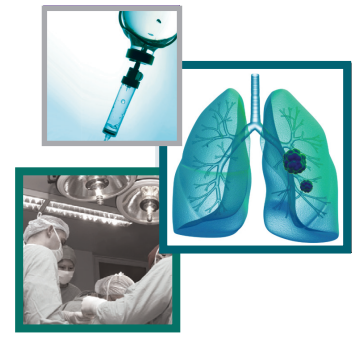

An update on current standards and clinical trials in systemic therapy for stage III NSCLC

Greg Durm*[,1] & Nasser Hanna[1]

Practice points

- Stage III NSCLC is a potentially curable disease though outcomes remain poor with 5-year overall survival rates of 14% in stage IIIA and 5% in stage IIIB.
- Concurrent chemoradiation remains the standard of care for patients with inoperable or unresectable disease.
- Surgical resection is an option for a subset of patients, and a multidisciplinary approach should be employed to determine which patients may benefit from this approach.
- Alternative strategies including induction, consolidation and maintenance chemotherapy have failed to demonstrate a clear improvement in overall survival.
- The addition of targeted agents in unselected patients has not shown benefit above standard chemoradiation.
- The use of antiangiogenic drugs has not improved survival outcomes and in some cases has proven harmful in combination with chemoradiation.
- Future studies will focus on the use of new immunotherapeutic agents as consolidation therapy and on using targeted drugs in selected patient populations.

Despite a number of recent breakthroughs in the treatment of metastatic NSCLC, the management of patients with stage III disease remains a challenge. The standard of care remains concurrent chemoradiation, and though a number of treatment strategies have been studied, no novel approach has clearly shown a consistent benefit. Future studies will focus on treatment with targeted therapies in selected patient populations and the use of novel immunotherapeutic strategies, such as checkpoint inhibitors, as consolidation therapy. This paper will review ongoing efforts to utilize innovative approaches to improve outcomes in this potentially curable subset of patients.

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Background

Lung cancer remains the leading cause of cancer-related mortality in the USA and in the world with an estimated 158,040 deaths in the USA and 1.7 million deaths globally in 2015 [1,2]. In the USA, it accounts for approximately 27% of all cancer deaths and has a higher overall mortality than breast, colorectal, and prostate cancers combined [1]. NSCLC makes up approximately 85% of all primary lung malignancies [3]. Outcomes in lung cancer are poor with a 5-year overall survival (OS) of 17.4%

KEYWORDS
• chemoradiation • clinical trials • NSCLC • stage III

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for all stages. This is largely due to the fact that only a small proportion (16%) of patients have localized disease at the time of diagnosis [4]. For patients with advanced disease and specifically those patients with stage III disease, the mortality outcomes are even worse with a 5-year OS of 14% in stage IIIA and 5% in stage IIIB NSCLC [5]. The challenges in treating this patient population include a median age >70 years and the presence of multiple cardiopulmonary comorbidities from prolonged tobacco exposure. Furthermore, a number of variables have been identified which predict for treatment toxicity and/or poor prognosis including decreased performance status, significant weight loss, presence of N3 disease, involvement of higher numbers of lymph node stations and a higher volume of lung parenchyma receiving greater than 20% (V_{20}) of radiation [6–8]. Despite these challenges, it is clear that a subset of these patients have curable disease and better approaches are needed to improve upon these results.

History of the treatment of stage III NSCLC

Until the 1980s, radiotherapy alone was the standard of care for patients with locally or regionally advanced NSCLC. This approach yielded 5-year OS rates of only 3–10%, and thus strategies utilizing additional treatment modalities were explored **(Table 1)** [9]. Two separate trials, the Cancer and Leukemia Group B (CALGB) and the Intergroup cooperative trial compared the use of radiation alone to the addition of sequential chemotherapy and found an OS advantage favoring the chemotherapy arm [9,10]. Subsequent trials compared sequential chemoradiation to concurrent chemoradiation and found a modest but significant OS benefit favoring concurrent therapy [11,12]. This was confirmed in a meta-analysis of six trials where concurrent chemoradiation demonstrated an absolute OS benefit of 5.7% at 3 years and 4.5% at 5 years [13]. Based on these findings, concurrent chemoradiation has become the standard of care in patients with inoperable or unresectable stage III NSCLC who are able to tolerate this therapy (adequate end organ function, absence of significant comorbidities, V_{20} <35%).

Although concurrent chemoradiation is the accepted standard of care for fit patients with inoperable or unresectable stage III NSCLC, the optimal regimen and duration of therapy have not yet been fully established. A number of platinum-based combinations have been tested previously, including a platinum agent plus mitomycin and vindesine, vinorelbine, irinotecan, vinblastine, etoposide, paclitaxel, nab-paclitaxel, docetaxel and pemetrexed [11–12,19,24–25,31–32]. Currently, there are few trials comparing these therapies directly. The West Japan Thoracic Oncology Group (WJTOG) conducted a study that compared second-generation to third-generation chemotherapy. This study randomized patients to receive mitomycin, vindesine, and cisplatin (MVP), carboplatin plus irinotecan, or carboplatin plus paclitaxel and showed no significant difference in overall survival but higher rates of hematologic and gastrointestinal

Table 1. Key historical studies in the treatment of inoperable or unresectable stage III NSCLC.

Treatment strategy	Key studies (group)	Comment	Ref.
Sequential chemoradiation	Dillman *et al.* (CALBG – 1996), Sause *et al.* (Intergroup–2000)	Established superiority of sequential chemoradiation over radiation alone	[9,10]
Concurrent chemoradiation	Curran *et al.* (RTOG – 2011), Furuse *et al.* (WJLCG – 1999), Auperin *et al.* (meta-analysis–2010)	Established superiority of concurrent over sequential chemoradiation	[11–13]
Induction chemotherapy	Vokes *et al.* (CALGB – 2007), Kim *et al.* (South Korea – 2007), Belani *et al.* (2005), Garrido *et al.* (2015), Descourt *et al.* (2011)	No trials have demonstrated improved survival with the use of induction chemotherapy	[14–18]
Consolidation/ maintenance chemotherapy	Hanna *et al.* (HOG – 2008),Ahn *et al.* (KCSG – 2015), Carter *et al.* (2012), Huber *et al.* (Germany – 2012), Tsujino *et al.* (pooled analysis – 2013)	No trials have demonstrated improved survival with the use of consolidation or maintenance chemotherapy	[19–23]
EGFR inhibitors	Kelly *et al.* (SWOG – 2008), Govindan *et al.* (CALGB–2011), Bradley *et al.* (RTOG – 2015)	The addition of the EGFR inhibitors gefitinib and cetuximab in unselected patients has not demonstrated improved OS	[24–26]
Anti-angiogenic agents	Wozniak *et al.* (2012), Spigel *et al.* (2010), Lu *et al.* (2010), Hoang *et al.* (ECOG – 2012)	Addition of antiangiogenic agents has proven to be either unsafe or of no benefit	[27–30]

CALGB: Cancer and Leukemia Group B; ECOG: Eastern Cooperative Oncology Group; HOG: Hoosier Oncology Group; KCSG: Korean Cancer Study Group; OS: Overall survival; RTOG: Radiation Therapy Oncology Group; WJLCG: West Japan Lung Cancer Group.

adverse events with MVP [31]. Another trial compared MVP to docetaxel plus cisplatin (DP) and found no significant difference in response rate (RR), progression-free survival (PFS) or OS for either group, though the DP arm showed a trend toward improved 2-year OS (p = 0.059). Higher rates of hematologic toxicity were again noted in the MVP arm while higher rates of grade 3 or higher radiation esophagitis were seen in the patients receiving DP [33]. Finally, a Phase III trial comparing cisplatin and pemetrexed with concurrent chemoradiation followed by maintenance pemetrexed versus cisplatin and etoposide with concurrent chemoradiation followed by consolidation chemotherapy has recently been completed. This trial demonstrated no difference in OS or PFS between the two arms; however, the cisplatin/pemetrexed arm had less grade 3/4 hematologic toxicity and lower rates of esophagitis [34]. Currently, the most commonly used regimens in the USA are carboplatin and paclitaxel or EP. There are no prospective trials comparing these two combinations; however, a retrospective analysis of Veterans Health Administration data was recently published which compared these two regimens in combination with concurrent radiotherapy. This study showed no difference in overall survival but a significant increase in toxicity with the use of EP [35]. A second systematic review was presented at the 2015 American Society of Clinical Oncology (ASCO) meeting and again showed no statistically significant difference in RR, PFS, or OS but increased rates of hematologic toxicity and nausea/vomiting with the use of EP [36].

A number of trials have explored the use of either induction or consolidation therapy in addition to concurrent chemoradiation. Unfortunately, none of these studies have been able to clearly demonstrate improved survival outcomes compared with chemoradiation alone, and many show increased toxicity. One of the largest studies of induction chemotherapy compared standard concurrent chemoradiation with weekly carboplatin and paclitaxel to two cycles of carboplatin and paclitaxel followed by the same regimen of concurrent chemoradiation. Overall survival rates demonstrated no significant difference between the two arms, and the induction chemotherapy arm showed greater hematologic toxicity and grade 4 adverse events [14]. A second randomized Phase III trial compared two cycles of induction cisplatin and gemcitabine followed by concurrent chemoradiation with carboplatin and paclitaxel to immediate concurrent chemoradiation with the same regimen. There was no significant improvement in OS for the induction arm, and there was actually improved PFS (7.5 vs 11.6 months; p = 0.04) for patients not receiving induction chemotherapy [15]. A number of other Phase II trials have also been completed looking at various regimens of induction chemotherapy. All of these trials were either nonrandomized or failed to show significant improvements in survival outcomes [16–18].

A number of Phase III trials have also explored the options of consolidation or maintenance therapy following concurrent chemoradiation. The Hoosier Oncology Group (HOG) completed a trial comparing EP followed by either consolidation docetaxel or placebo. This trial demonstrated no improvement in OS for the consolidation arm and showed higher rates of hospitalization and treatment-related death for patients treated with docetaxel [20]. Several trials have also looked at the role of maintenance chemotherapy following concurrent chemoradiation. A number of different regimens have been studied including cisplatin plus docetaxel [19], weekly paclitaxel [21] and cisplatin plus vinorelbine [22], but all of these trials failed to demonstrate improved OS in the maintenance chemotherapy arms. Finally, in a pooled analysis of the literature by Tsujino *et al.*, 41 studies (7 Phase III and 34 Phase II) of consolidation chemotherapy were analyzed and found no significant difference in median overall survival (19 vs 17.9 months; p = 0.4) for consolidation therapy compared with no additional treatment [23]. With current available drugs, it appears that a plateau for mortality outcomes has been reached using current strategies in stage III NSCLC, and concurrent chemoradiation with a platinum-based regimen remains the standard of care in this disease.

Better patient selection and supportive care are likely to continue to marginally improve patient outcomes in this setting, and one way to enhance this is through improved initial staging. Both PET-computed tomography (PET-CT) and endobronchial ultrasound (EBUS) have enhanced the ability to provide accurate staging to newly diagnosed patients. A study presented at the World Conference on Lung Cancer (WCLC) in 2015 compared the effectiveness of these two modalities for staging of mediastinal lymph nodes. The sample size in this study was small, but it showed discordant results between

PET-CT and EBUS in 33% of the participants, with three patients being upstaged and two being downstaged following EBUS [37]. This suggests that utilization of EBUS may be necessary to plan for initial therapy in NSCLC patients, particularly when planning radiation fields or surgery. In regards to supportive care, one controversy is the use of prophylactic cranial irradiation (PCI) for stage III NSCLC patients following definitive treatment. Two studies looked at PCI in stage III patients in this setting and both showed a significant decrease in the rate of brain metastases but no improvement in OS [38,39]. A separate meta-analysis of all NSCLC patients again demonstrated a significant decrease in brain metastases and actually showed a statistically significant improvement in 1-year OS (RR: 1.09; $p < 0.001$) for the PCI group. However, the number needed to treat to save one life was 100, and the quality of the included studies was poor [40]. Thus, the question of whether PCI should be used and in which patients remains to be answered.

Role of surgery

Trimodality therapy with chemoradiation followed by surgical resection is another potential option for a subset of patients with stage III disease. An early trial by Albain *et al.* in patients with stage IIIA(N2) disease randomized patients to receive concurrent chemoradiation to 61 Gray (Gy) versus concurrent chemoradiation to 45 Gy followed by surgical resection. The surgical arm demonstrated greater PFS (mPFS 12.8 vs 10.5 months; 5-year PFS 22.4 vs 11.1%) but failed to show statistically significant improved OS (mOS 23.6 vs 22.2 months; 5-year OS 27.2 vs 20.3%). An exploratory analysis did show improved OS for patients undergoing lobectomy over chemoradiation alone, but this was not demonstrated in those undergoing pneumonectomy. Furthermore, there were much higher rates of treatment-related death in the surgical arm (7.9 vs 2.1%), most of which were in pneumonectomy patients [7]. Another study randomized patients with stage IIIA(N2) disease to either surgical resection versus radiotherapy following induction chemotherapy. There was no statistical improvement in either PFS (9 vs 11.3 months) or OS (16.4 vs 17.5 months) in the surgery versus radiation arms. An exploratory analysis in this study also showed improved outcomes for patients undergoing lobectomy in comparison to pneumonectomy [41]. A recently presented abstract at ASCO 2014 randomized operable stage IIIA and selected IIIB patients to either surgical resection or completion of definitive concurrent chemoradiation. All patients initially underwent induction chemotherapy with three cycles of cisplatin and paclitaxel followed by concurrent chemoradiation with cisplatin and vinorelbine. Patients were randomized to receive radiation to 45 Gy followed by resection or to complete concurrent chemoradiation to a risk-adapted 65/71 Gy. The trial closed early due to slow accrual and did not demonstrate a significant difference in overall survival between the two groups. However, both groups showed excellent 5-year OS rates, 40.6% in the chemoradiation arm and 44.2% in the surgical arm [42]. Lastly, a retrospective analysis presented at the WCLC in 2015 looked at outcomes in patients receiving chemoradiation alone versus patients receiving chemoradiation followed by lobectomy or pneumonectomy. The median and 5-year OS rates were highest in the two surgical arms with 5-year survival rates of 44 and 33% in the lobectomy and pneumonectomy arms, respectively, compared with 14% in the chemoradiation alone arm [43]. Approximately 92% of the patients in this analysis received chemoradiation alone, and the surgical patients likely represent a highly selected group. However, despite the selection bias and retrospective nature of the study, this data in combination with previous trials suggests that a subgroup of patients derive clinical benefit from surgery following chemoradiation. Appropriate patient selection requires a multidisciplinary approach that includes medical oncology, thoracic surgery and radiation oncology input to decide the best treatment approach for each individual patient.

Incorporation of novel agents

Initial attempts at incorporating novel targeted therapies into standard chemoradiation have thus far yielded disappointing results. In the metastatic setting, success has been seen using tyrosine kinase inhibitors (TKI) targeted against EGFR, with the majority of the benefit seen in patients with known *EGFR* mutations. Efforts to utilize these agents in the treatment of stage III lung cancer have not been favorable; however, reported trials to date have been in unselected patient populations. The Southwest Oncology Group (SWOG) completed a Phase III trial treating unselected patients with stage III NSCLC with standard chemoradiation followed by mandatory docetaxel consolidation. All patients were then randomized to receive either additional gefitinib consolidation or placebo. This study actually showed a statistically significant

difference in OS favoring the placebo arm [26]. A recently published study by the Radiation Therapy Oncology Group (RTOG) included four arms and compared different radiation doses (60 vs 74 Gy) as well as the addition of cetuximab to standard chemoradiation with carboplatin and paclitaxel. The results of the study favored the 60 Gy arm and the addition of cetuximab provided no additional survival benefit [25]. A separate Phase II trial looked at the feasibility of concurrent chemoradiation using cisplatin and pemetrexed with or without the addition of cetuximab. Overall survival was similar in both arms suggesting no benefit with the addition of cetuximab [24].

Several studies have also looked at the safety and efficacy of adding antiangiogenic therapies to standard chemoradiation. A pilot study looked at the safety of incorporating bevacizumab into consolidation therapy in combination with docetaxel following standard chemoradiation with EP. Patients were risk-stratified into high- and low-risk strata for adverse events. The high-risk stratum was closed secondary to adverse events including pneumonitis and fatal hemoptysis, and the low-risk stratum closed secondary to poor accrual [27]. Another study looking at the safety of adding bevacizumab to carboplatin and pemetrexed given concurrently with thoracic radiation closed early secondary to safety concerns after enrollment of only five patients. Two patients developed tracheoesophageal fistula with one resulting in death [28]. Two studies utilizing other antiangiogenic therapies, thalidomide and an experimental drug (AE-491), failed to show improvement in OS, and the addition of thalidomide also demonstrated increased toxicity [29,30]. In summary, the addition of antiangiogenic agents to chemoradiation in stage III NSCLC has thus far been ineffective and potentially even unsafe.

Despite disappointing results with the initial implementation of EGFR TKIs in stage III NSCLC, the scientific rationale for this approach remains intriguing. Cell lines with overexpression of wild-type EGFR activity have demonstrated increased radiation resistance. A few mechanisms for the decreased sensitivity to radiation have been suggested including the influence of EGFR on DNA repair mechanisms, suppression of apoptosis through activation of the PI3K/AKT pathway, and enhanced malignant cell repopulation mediated through radiation-induced phosphorylation of EGFR and activation of the RAS and STAT pathways [44]. Furthermore, cell lines harboring known *EGFR* mutations exhibit enhanced sensitivity to radiation therapy compared to *EGFR* wild-type cell lines, likely secondary to the inability to bind key DNA repair enzymes [44,45]. Similarly, *in vitro* studies exploring the interaction of ALK inhibition and radiation suggest that, in mouse models harboring the EML4–ALK fusion, treatment with crizotinib resulted in effective blockade of downstream signaling pathways. Furthermore, the combination of crizotinib and radiation resulted in greater tumor growth inhibition than either treatment alone suggesting that crizotinib may act as a radiosensitizer in patients with EML4–ALK fusions. These effects were not observed in the mouse model without an EML4–ALK fusion [46].

Based on these preclinical studies, further investigation into EGFR and ALK inhibition in stage III NSCLC patients treated with chemoradiation is warranted. Currently, a study of either erlotinib or crizotinib as induction therapy in stage III NSCLC is accruing patients (NCT01822496) **(Table 2)**. This study will enroll approximately 234 patients to one of four arms which will be stratified based on EGFR and ALK status. Patients with an activating *EGFR* mutation or *ALK* rearrangement will be randomized to receive erlotinib or crizotinib, respectively, as induction therapy or to undergo immediate chemoradiation with a platinum-based regimen. For patients receiving induction, those not responding at 6 weeks will begin chemoradiation, and those responding will continue for up to 12 weeks of therapy. 2 weeks following completion of induction, these patients will then receive identical chemoradiation. The hypothesis is that targeted induction therapy in a selected population should improve radiation sensitivity, and therefore patients randomized to erlotinib or crizotinib should have improved progression-free survival compared with those on standard chemoradiation alone.

In addition to EGFR and ALK, other molecular targets may be of interest in stage III NSCLC. The *RAS* oncogene has been shown to increase radiation resistance, and while previous efforts to directly target RAS have been disappointing, inhibition of downstream pathways may be feasible [47]. Currently, a Phase I study incorporating the MEK inhibitor, Trametinib, into standard chemoradiation for patients with stage III NSCLC that harbors a *KRAS* mutation is underway (NCT01912625). Furthermore, preclinical data suggest that PI3K may be a mediator of RAS-induced radiation resistance, and thus, future trials investigating the efficacy of PI3K inhibition in *KRAS*-mutant NSCLC may be warranted.

Table 2. Key ongoing or planned clinical trials in unresectable or inoperable stage III NSCLC.

Type of therapy	Phase/schema	NCT registry number
Targeted agents	Randomized Phase III: – Arm 1 – Erlotinib followed by CCRT (*EGFR* mutation +) – Arm 2 – CCRT only (*EGFR* mutation +) – Arm 3 – Crizotinib followed by CCRT (*ALK* TL +) – Arm 4 – CCRT only (*ALK* TL +)	01822496
	Phase I: – Chemoradiation with trametinib followed by consolidation chemotherapy in *KRAS*-mutant patients	01912625
Immunotherapy	Randomized Phase II (following standard CCRT): – Arm 1 – DRibbles vaccine and HPV vaccine – Arm 2 – DRibbles vaccine, HPV vaccine, and Imiquimod – Arm 3 – DRibbles vaccine, HPV vaccine, and GM-CSF	01909752
	Single arm Phase II (following standard CCRT): – Tecemotide with Bevacizumab	00828009
	Single arm Phase II (following standard CCRT): – Pembrolizumab (PD-1 inhibitor)	02343952
	Randomized Phase III (following standard CCRT): – Arm 1 – MEDI4736 (PDL-1 inhibitor) – Arm 2 – Placebo	02125461
Miscellaneous	Randomized Phase II: – Arm 1 – CCRT followed by consolidation chemotherapy – Arm 2 – Metformin × 14 days followed by CCRT and metformin followed by consolidation chemotherapy and metformin	02186847

ALK TL: ALK translocation; CCRT: Concurrent chemoradiation; HPV: Human papilloma virus; NCT: National Clinical Trial.

Recent trials involving immunotherapeutic targeting of T-cell regulation and activation have demonstrated encouraging results in metastatic NSCLC. Phase III trials have been completed in both squamous [48] and nonsquamous histologies (CheckMate-057 Trial, Bristol-Myers Squibb press release 17 April 2015) and demonstrate significantly improved OS for the PD-1 inhibitor, nivolumab, when compared to docetaxel in previously treated patients. This led to the FDA approval of nivolumab for previously treated patients with squamous NSCLC. Other checkpoint inhibitors such as pembrolizumab (PD-1 inhibitor) and ipilimumab (CTLA-4 inhibitor) have also shown activity in early phase trials in advanced NSCLC [49,50].

Immunotherapy for stage III NSCLC is also being studied with some initial success. At the ASCO meeting in 2013, the results of a Phase III randomized trial of consolidation immunotherapy was reported. In that trial, patients with inoperable or unresectable stage III NSCLC were treated with standard chemoradiation (65% concurrent and 35% sequential) and then randomized to receive tecemotide, a MUC1 antigen-specific cancer vaccine, or placebo. The study failed to meet its primary endpoint of improved OS, however, a preplanned subgroup analysis showed improvement in median OS from 20.6 to 30.8 months (HR: 0.78; p = 0.016) for the group receiving concurrent chemoradiation in combination with the vaccine [51]. A second trial of tecemotide has recently completed accrual through the Eastern Cooperative Oncology Group (ECOG). This trial (NCT00828009) enrolled patients with inoperable nonsquamous stage III NSCLC and treated them with concurrent chemoradiation with carboplatin and paclitaxel followed by two cycles of consolidation chemotherapy. Following consolidation, patients who have not progressed were treated with a single dose of cyclophosphamide followed by the combination of tecemotide and bevacizumab. The treatments were repeated every 21 days for up to 34 cycles in the absence of progression or unacceptable toxicity. The primary endpoint is to determine whether bevacizumab and tecemotide can be given safely in the maintenance setting following concurrent chemoradiation.

Given the success in the metastatic setting, the utilization of PD-1/PDL-1 inhibitors in stage III NSCLC is also a very attractive approach. Preclinical data suggest that ionizing irradiation (IR) causes direct cytotoxic cell death and tumor antigen release, upregulates PDL-1

expression in the tumor microenvironment and enhances tumor control both locally and distally [52,53]. However, it is clear that treatment failures following radiation or chemoradiation alone are common in locally advanced NSCLC, and thus new approaches are needed to improve upon these strategies. The use of PD-1/PDL-1 inhibitors is a rational approach in this setting as the increase in tumor antigen release may be synergistic with the increased activation of the T-cell response provided by PD-1 blockade. Furthermore, preclinical data support this as the addition of PDL-1 therapy to IR enhanced the cytotoxic T-cell response and synergistically altered the tumor microenvironment [52].

Trials testing the efficacy and safety of PD-1/PDL-1 inhibitors following chemoradiation for stage III NSCLC are currently underway. A Phase III trial testing MEDI4736, an antibody to PDL-1, in a Phase III industry-sponsored trial (PACIFIC) will enroll 702 patients across 100 international sites (NCT02125461). Patients with unresectable stage III NSCLC will be treated with concurrent chemoradiation with platinum-based chemotherapy, and if not progressing, they will be randomized (2:1) to receive either MEDI4736 or placebo for up to 1 year as consolidation. Overall survival is the primary endpoint. Another trial evaluating pembrolizumab, an antibody to PD-1, as consolidation therapy following concurrent chemoradiation is also currently accruing patients. This trial, conducted through the Hoosier Cancer Research Network (HCRN) is a Phase II, single-arm trial of pembrolizumab following standard chemoradiation in inoperable or unresectable stage III NSCLC (NCT02343952). All patients will receive standard concurrent chemoradiation, and those patients who do not progress will receive consolidation pembrolizumab every 3 weeks for up to 1 year. The primary endpoint is time to distant relapse, and an exploratory analysis will look at the correlation of PDL-1 status to patient outcomes. A third study, a randomized trial with nivolumab after chemoradiation, is also currently under development.

Conclusion & future perspective

The treatment of stage III NSCLC continues to present important challenges in the world of lung cancer. The 5-year survival rates for these patients are poor secondary to a number of factors including older age, presence of multiple comorbidities and toxicity of treatment. The standard of care remains concurrent chemoradiation in patients with inoperable or unresectable disease who are fit enough to tolerate this therapy. Surgery remains an option for a small subset of patients with stage III disease. Despite testing multiple strategies including induction, consolidation, maintenance, multiple different chemotherapy regimens and the addition of targeted agents in unselected patients, advances in this population have been difficult to demonstrate. Future investigations will focus on incorporating targeted therapies, particularly EGFR- and ALK-directed, into selected patient populations. Furthermore, much attention will be given to the addition of immunotherapeutic agents in combination with chemoradiation given the success of these agents in the metastatic setting. Lastly, more work is needed to identify further molecular targets that may potentially be exploited in this disease. It is encouraging to see considerable advances in the treatment of metastatic NSCLC, and hopefully, utilizing these innovations in the stage III setting will increase the success rate in this potentially curable population.

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Review

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Biomarkers in Medicine

The challenge of small lung nodules identified in CT screening: can biomarkers assist diagnosis?

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Various biomarkers have been developed as noninvasive tests to indicate the presence of lung cancer in asymptomatic persons, and in particular to provide evidence as to whether indeterminate lung nodules detected by screening are malignant. We performed an overview of the range of biomarkers reported in the literature and described those that can complement low-dose computed tomography screening. Several have promising sensitivity and specificity. However to our knowledge, only three techniques have reached the prospective screening phase (phase 4) of the five-phase biomarker development process. Two miRNA signatures (the miR-Test for serum and the miRNA signature classifier test for plasma) are being assessed in prospective screening trials, as is the EarlyCDT-Lung test based on autoantibodies. All will need to undergo prospective studies to determine their ability to improve outcomes before they can become an established adjunct to lung cancer control strategies.

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Lung cancer is the leading cause of cancer death worldwide. Incidence continues to grow among women in developed countries and among both sexes in developing countries [1]. Single-arm and randomized studies on early lung cancer detection with low-dose computed tomography (LDCT) without contrast have shown that the technology is highly sensitive for detecting small lung nodules, with short examination times and limited radiation exposure [2–5]. The lung cancer detection rate is high and stable over time in high-risk populations, with recall rates, false-positive rates and estimated overdiagnosis rates all acceptable in well-designed studies [6]. Costs are also acceptable [7]. To limit the number of useless investigations and consequent risk of morbidity in screened subjects, while ensuring that that few cancers escape early detection, standardized algorithms for managing indeterminate lung nodules have been developed [8–11].

Importantly, the US National Lung Screening Trial showed that LDCT screening reduces mortality compared with standard x-ray [12]. However this trial was characterized by high recall and high false-positive rates [13], attributable to suboptimal study design, particularly lack of a pre-established diagnostic algorithm used by all centers [14].

The performance of other single-arm or randomized studies was better [6,9,10]. For example, in the single-arm COSMOS study, the diagnostic algorithm was noninvasive, with no routine CT-guided transthoracic biopsy, and indication for surgery based on nodule size, volume doubling time (VDT), and standard uptake values (SUV) on PET-CT. COSMOS had among the lowest false-positive and false-negative rates (13%) [10]. The Danish screening study found that a combination of VDT and standard uptake value predicted the presence of lung cancer with impressive accuracy [11].

Future Medicine part of fsg

The need for biomarkers to complement low-dose computed tomography screening

In view of its ability to reduce lung cancer mortality compared with CXR screening [12], LDCT screening for high-risk individuals is now recommended [15,16] and is being implemented in the USA. It is hoped that large-scale LDCT screening will also be implemented in Europe; however, the process is slowed down by the controversial results in terms of mortality reduction of randomized trials in Europe as the Dante study [17]. In the meantime it is important to further improve screening efficacy, particularly by reducing the number of potentially harmful invasive procedures carried out to diagnose benign disease. It is in this area that disease biomarkers may be particularly useful, and various biomarkers are being assessed for their ability to diagnose lung cancer in high-risk asymptomatic individuals. Such markers could be used to select those who should preferentially undergo screening, potentially improving screening compliance, and also sparing those with a negative profile (and thus low risk of lung cancer) from radiation exposure. An effective biomarker could also be incorporated in a risk model that, incorporating epidemiologic and clinical risk factors, might more accurately select which high-risk individuals should be screened [18].

Similarly, biomarker data could be used in a predictive model with CT findings (presence and characteristics of nodules, presence of emphysema) and epidemiologic and clinical risk factors, to stratify individuals according to the probability of being diagnosed with lung cancer at repeat screening [18]. In this context, a biomarker would provide additional evidence as to whether a suspicious screening-detected nodule was malignant or not, reducing the number of false positives at surgery or surgical biopsy.

Further, since LDCT screening is relatively insensitive to centrally located lesions and to small aggressive cancers (the latter typically diagnosed between screening rounds – interval cancers – when they become symptomatic), an effective biomarker would also signal the presence of cancer before scheduled CT screening, prompting immediate diagnostic workup.

In the screening setting however, a biomarker would not necessarily be required to distinguish indolent from aggressive disease since, once the biomarker profile indicates lung cancer, CT would be used to find the nodule, and nodule characteristics (type, shape, VDT) would indicate disease aggressiveness. Nevertheless, there is evidence (reviewed below) that the 'strength' of some biomarkers does correlate with disease aggressivity. Different molecular markers (gene signatures) have been described from the tissue with the main aim to stratify patients according to prognostic profile and identify candidate to adjuvant treatment [19]. Obtaining the same information from blood before surgery would be of great utility to decide therapeutic strategies. Finally, the use of biomarkers to detect a cancer years before a nodule is evident on CT may not be particularly useful, as the long-term stress to the patient may outweigh the benefit, particularly since a biomarker diagnosis without a nodule is unlikely to have any impact on clinical management.

In general, substances or cells secreted by the tumor (or tumor microenvironment [20]), and not secreted by healthy tissue, that are reliably detected in blood (or urine or sputum) at contained costs, are candidates for biomarkers. Five phases of biomarker development have been identified, these are: discovery; clinical development and validation; retrospective longitudinal validation; prospective screening; and impact on cancer control [21] Regarding the US federal cost reimbursement, an issue that is emerging is that CT screening is going to be compensated at a rate perhaps under US$200, which has negative corresponding cost implications for biomarkers studies.

Biomarkers arising from the host immune response to cancer

A Spanish group has carried out extensive investigations of c4d, a stable degradation product of complement activation [22]. The group showed that lung cancer cells activate the classical complement pathway, part of the innate immune system, and generate c4d. Plasma from patients with early lung cancer had significantly higher levels of c4d than plasma from controls (patients with nonmalignant respiratory disease). Cancer patients with higher levels of c4d (>3 μg/ml) had significantly worst prognoses than those with low c4d levels. In all but one of the 25 lung cancer patients with high preoperative c4d levels, levels reduced after surgical removal of the cancer providing evidence that plasma c4d levels are related to cancer presence. When tested on 190 asymptomatic individuals enrolled in a CT screening program, plasma c4d levels were significantly higher in individuals with lung cancer than those without, suggesting a potential role of c4d to predict the lung cancer in the screening setting. However, the area under the receiver operating characteristic (ROC) curve (plotting sensitivity against 1-specificity) was 0.735, symptomatic of relatively low test sensitivity.

Another serum protein of inflammation, from the class of CRP, has been identified in early lung cancers with [23]. With a cutoff of 4.5 ng/ml, specificity of PTX3 was 0.80 and sensitivity was 069 with an AUC of 83.5.

It is known that cancer patients produce autoantibodies to mutated, aberrant or overexpressed proteins

produced by cancer cells. The autoantibody signature (EarlyCDT-Lung test) for lung cancer developed by groups collaborating with JF Robertson and the Oncimmune company is based on a panel of seven autoantibodies expressed in patients with lung cancer and other diseases [24–26] and detected by simple ELISA tests. The panel has been validated in a large series of patients (with early or late stage non-small-cell lung cancer [NSCLC] or small-cell lung cancer) and controls. However, while specificity is in the range 87–91%, sensitivity is only 41–44% and this is a major drawback in the screening setting since a negative test result does not rule out lung cancer. A prospective screening study is ongoing in Scotland further to assess the role the EarlyCDT-Lung test in the early diagnosis of lung cancer. Ten thousand high-risk smokers are being recruited. Half will be given the test and if positive will be followed by LDCT at intervals for 2 years [27]. The other half will not have the test but current standard of care instead. Outcomes and cost of care (including the test) will be compared in the two groups.

Zhong *et al.* [28] developed a panel to detect NSCLC-related autoantibodies (and hence NSCLC) in plasma samples. A cDNA library, previously enriched in cDNAs coding for NSCLC-associated proteins, was expressed in a T7-phage system. The phage-expressed proteins were screened with patient plasma to identify those recognized by tumor-associated antibodies. The immunogenic proteins were ranked for their reactivity to plasma samples from 23 stage I patients and 23 risk-matched controls. All samples were used as a training set to identify the combination that best distinguished patients from control samples. The panel of five markers thus identified was then used to predict disease probability in 102 samples from the Mayo Clinic CT Screening Trial, consisting of 56 risk-matched non-cancer samples, six prevalence cancer samples and 40 incidence cancer samples drawn 1–5 years prior to cancer detection at first screening round. The panel of markers correctly predicted all six prevalence cancers, 32 of 40 cancers in samples taken before radiographic detection, and 49 of 56 noncancers.

A blood-based proteomic signature for lung cancer has been developed by researchers at SomaLogic, Inc. [29]. The aptamer-based SOMAscan V2 system was employed to screen for 1033 proteins. Elaborate measures were used to exclude bias due to preanalytic sample variation in the discovery phase, and increase the chances that the initially identified panel would be confirmed in validation phases. The eventual outcome was a Random Forest model of seven proteins. Areas under ROC curves for this panel were 0.85 for all training samples (68% adenocarcinoma, 32% squamous cell carcinoma) and 0.93 for squamous cell carcinoma. The panel was further assessed by making blinded predictions on a reference set of cancer cases and radiologically negative smokers as controls. The signature appears promising but has suboptimal sensitivity (33–50% sensitivity for stage I adenocarcinoma; 64–93% for squamous cell carcinoma) [29].

miRNA biomarkers

miRNAs are short noncoding RNAs that modulate gene expression by cleaving, destabilizing or otherwise impeding the translation of mRNAs. MiRNA expression is deregulated in a variety of diseases including cancer and it has been shown that specific expression patterns of serum miRNAs occur in patients with lung cancer, colorectal cancer and diabetes [30]. Furthermore miRNAs are remarkably stable in human blood where they are protected from endogenous RNAse activity [30].

Several groups have published studies on the use of miRNA signatures to diagnose NSCLC. Chen *et al.* [30] identified two serum miRNAs (miR-25 and miR-223) specific for NSCLC and further validated them in independent samples of 75 healthy donors and 152 cancer patients. Shen *et al.* [31] found several miRNAs that were aberrantly expressed in early-stage NSCLC. A logistic regression model incorporating four of these (miR-21, miR-126, miR-210 and miR-486–5p) had 86.22% sensitivity and 96.55% specificity in distinguishing NSCLC patients from the healthy controls. The panel had 73% sensitivity and 97% specificity in identifying stage I disease, and higher sensitivity in diagnosing adenocarcinoma (92%) than squamous cell carcinoma (82%).

Zheng *et al.* [32] found that levels of miR-155, miR-197 and miR-182 were significantly higher ($p < 0.001$) than controls in the plasma of lung cancer patients, including those with stage I disease. The combination yielded 81% sensitivity and 87% specificity in discriminating patients from controls. Levels of miR-155 and miR-197 were significantly higher in metastatic than nonmetastatic patients, and significantly decreased in responsive patients during chemotherapy.

In 2012 Hennessey *et al.* [33] reported that, in a training set of serum samples from 30 treatment-naive NSCLC patients and 20 healthy controls, levels of miR-15b and miR-27b distinguished patients from controls with sensitivity, specificity, positive predictive value and negative predictive value of 100%. Further testing on 55 NSCLC and 75 healthy controls showed that the miRNA pair predicted NSCLC with a specificity of 84% (95% CI: 0.73–0.91), sensitivity of 100% (95% CI: 0.93–1.0) and negative predictive value of 100%.

It should be noted that while all the above-reported results [28–31] appear promising, it is unclear whether

the miRNA signatures will be useful for identifying cancer in asymptomatic persons to thereby anticipate diagnosis.

Two cancer institutes in Milan – the National Cancer Institute (INT) and the European Institute of Oncology (EIO) – have independently developed different miRNA signatures to detect lung cancer in high-risk asymptomatic individuals. The EIO signature was developed in serum [34], the INT signature was developed in plasma [35].

The EIO signature, initially of 34 miRNAs, was derived by analyzing NSCLC cases and matched noncancer controls enrolled in the COSMOS study (training set) [10]. At the time of serum collection, all NSCLC cases were asymptomatic, and cancer was detected as a consequence of enrollment in COSMOS. The signature was validated on separate cohorts of COSMOS subjects (testing set), and further validated on preoperative serum samples from symptomatic lung cancer patients not part of the COSMOS study, matched to preoperative samples from pulmonary hamartomas patients. When the risk index produced by the signature was stratified by clinical and pathological variables, it remained a strong predictor of lung cancer in all patient subgroups considered. In the training set, the risk algorithm had an accuracy of 78% and area under the ROC curve of 0.92. In the testing set, accuracy was 80% and area under the ROC curve was 0.89.

Signature performance in 33 patients with benign lung nodules on baseline LDCT who did not develop lung cancer during the entire COSMOS follow-up, did not differ from that in 30 noncancer controls with no nodules, indicating that the predictor 'saw' cases with benign nodules as normal.

For 13 patients who developed lung cancer over a year after an initially negative LDCT, and had serum samples both before and after disease onset, the average risk index was significantly greater for samples collected after disease onset; thus the signature was sensitive to the conversion from normal to malignant state. When the signature was tested for cancer specificity, it was found that risk indexes for samples from breast cancer patients clustered with those of healthy plus benign nodule-samples, and were distinct from lung cancer cases [34].

In a subsequent large-scale validation study [34,36] the EIO's original 34 miRNAs were first reduced to 13 miRNAs, and the test (now called the miR-Test) was found to have the same performance as the original signature. miR-Test sensitivity was 79.2% (95% CI: 67.7–90.7%) and specificity was 75.9% (95% CI: 73.3–78.5%). Overall, 820 of the 1115 (73.5%) individuals tested were miR-Test-negative, including 810 of 1067 without lung cancer and 10 of 48 with lung cancer. Negative predictive value was >99%, suggesting that persons who were miR-Test-negative could safely avoid LDCT. The sensitivity and negative predictive value of miR-Test were comparable with LDCT alone, suggesting that the miR-Test could be used for first-line screening. The test had lower specificity than LDCT; however, since all miR-Test-positive cases would undergo CT to confirm diagnosis and localize the lesion for surgery, this would not matter.

The Milan INT study [37] analyzed miRNA expression in diseased and healthy lung tissue as well as in plasma samples. Plasma samples were collected from patients (1–2 years before disease onset) and disease-free smokers enrolled in two independent LDCT screening studies; they provided a signature involving 24 miRNAs that had good diagnostic and prognostic potential. Thus, like the IEO signature, the INT signature identifies lung cancer in asymptomatic persons. A further validation [37] of the INT signature (now called the miRNA signature classifier, or MSC) on 939 participants of the randomized MILD screening trial [38] showed that the MSC had 87% sensitivity and 81% specificity across both MILD arms (LDCT and observation), and 88% sensitivity and 80% specificity in the LDCT arm. Negative predictive value was 99%. Use of MSC together with LDCT resulted in a fivefold reduction in false-positives on LDCT.

It is interesting that only five of the 13 miRNAs comprising the EIO's miR-Test are present among the 24 miRNAs of the INT's MSC, while only nine miRNAs are common to the EIO's original signature of 34-miRNAs and the INT's MSC [19]. The common miRNAs may perhaps be those that fundamentally distinguish individuals with lung cancer from those without the disease, while the differences could in part be due to the fact that one signature was developed in serum and the other in plasma. However, it is also noteworthy that there is little or no overlap between the miRNAs of any of the miRNA signatures reported as distinguishing lung cancer patients from nonpatients [30–35]. The problem is discussed by Chen *et al.* [30], but reasons for these differences are unclear. Both the miR-Test and the MSC test are currently being validated in prospective screening trials (COSMOS II and bioMILD, respectively) and have thus reached phase 4 of the 5-phase development process outlined in the JNCI Commentary [21].

Other potential biomarkers

Other biomarkers are under evaluation although they are at less advanced stage of validation than the miRNA signatures discussed above. The LuCED Lung Test [39] performs an automated 3D morphological analysis of epithelial cells from sputum samples. Each cell is assessed for 594 morphology-based features to quanti-

Table 1. Description of the characteristics of the principles diagnostic markers of lung cancer for early detection phase.

Category	Phase of validation	Sensitivity (%)	Specificity (%)	Ref.
Host immune response				
C4D	Case–control screening	NA (AUC 0.73)		[22]
Pentraxin	Case–control screening	80	69	[23]
Autoantibody	Prospective screening	87–91	41–44	[25]
SOMAscan	Prospective screening	33–93	71–90	[29]
MicroRNA				
MicroRNA US	Validation set	84	100	[33]
MicroRNA INT	Prospective screening	88	80	[37]
MicroRNA EIO	Prospective screening	72.2	77.8	[36]
Tumor cells				
Sputum	Case–control screening	91.8	95.2	[39]
Blood	Case–control screening	100	100	[40]

tatively assess whether tumor cells are present and then characterize them by tumor type. Sputum samples from patients with known benign and malignant outcomes were used to assess LuCED as a possible adjunct to a suspicious finding on LDCT or as independent screening test for lung cancer. After operator review, the LuCED test had 91.8% sensitivity and 100% specificity. The main limitation was that a high proportion of sputum samples turned out to be inadequate.

A filtration technology called Isolation by Size of Epithelial Tumor Cells has been developed to detect circulating tumor cells (CTCs) in blood. A recent study looked for CTCs using Isolation by Size of Epithelial Tumor Cells in persons without clinically detectable lung cancer, who were undergoing LDCT screening, as a first step to identifying a new marker for early lung cancer diagnosis [40]. A total of 245 persons were examined: 168 (68.6%) with chronic obstructive pulmonary disease (COPD) and 77 (31.4%) without COPD; the latter group comprised 42 control smokers and 35 nonsmoking healthy individuals. In the five patients diagnosed with lung cancer – all with COPD – CTCs were identified 1–4 years before a nodule was identified on LDCT. CTCs were not detected in smoker controls or nonsmoking healthy individuals. Further studies are required to confirm these preliminary findings [40].

Conclusion

Three promising circulating biomarkers are in an advanced phase of validation for the noninvasive diagnosis of lung cancer in asymptomatic high-risk individuals. These are the miR-Test [34] and the MSC test [35], both miRNA signatures, and the EarlyCDT-Lung test [23] based on autoantibodies (Table 1). Validated biomarkers are urgently needed to more accurately select high-risk individuals for LDCT screening and assist the diagnosis of indeterminate lung nodules detected during screening. Biomarkers might usefully be incorporated into risk models to select persons for screening, or stratify those being screened according to lung cancer risk.

Future perspective

It is expected that effective biomarkers to assist the early diagnosis of lung cancer in the screening setting will become available within a few years. Biomarkers have the potential to reduce screening costs, reduce the number of false positives on surgery/surgical biopsy of screening-detected nodules, and reduce the number of overdiagnosed lung cancers. They may therefore be a crucial element in cost–effective screening programs to reduce lung cancer mortality. The creation of networks of investigators using pooled databases and biospecimen repositories, such as the US Early Detection Research Network, will facilitate the future validation and comparison of biomarkers in blood or airways to detect lung cancer in asymptomatic high-risk cohorts.

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Executive summary

- Low-dose computed tomography (LDCT) screening detects lung cancer at an early stage, improving survival and reducing mortality.
- Biomarkers promise to improve the selection of high-risk persons for LDCT screening, reducing screening costs.
- Biomarkers also promise to render the preoperative diagnosis of LDCT-detected lung nodules more accurate, reducing false positives at surgery/surgical biopsy.
- Several biomarkers have shown promising initial results.
- Two circulating miRNA signatures (miR-Test and miRNA signature classifier) and a circulating marker of compliment activation (c4d) have reached the prospective testing stage.

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