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The potential of trefoil proteins as biomarkers in human cancer

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Knowledge about the molecular etiology of human cancer underwent a seismic shift 30 years ago with the realization that viral oncogenes have cellular counterparts. Since then, the identification and characterization of oncogenes that are activated by enhanced expression or mutation, and of tumor suppressor genes that are inactivated by reduced expression or mutation, has dominated research. This molecular revolution has included increasingly sophisticated gene expression analyses that have led to the concept of gene signatures. Such approaches reached a zenith with the application of next-generation sequencing to the characterization of cancer cell genomes and transcriptomes.

Most human cancers are solid tumors that arise from glandular epithelia; these adenocarcinomas account for the preponderance of cancer-associated morbidity and death worldwide. It is instructive to consider the impact of the above endeavour on the clinical management and outcome of patients with adenocarcinoma. There are examples, notably in colorectal, breast and lung cancer, of consequent introduction of effective treatments and useful biomarkers, however, for the vast majority of patients, treatment and survival have not been affected; improved outcome is explained by earlier diagnosis, reduced surgical morbidity, the introduction of adjuvant therapy and advances in systemic cytotoxic and endocrine therapies. Implementation of effective biomarker-dependent patient stratification should be a major goal of molecular medicine.

It is accepted that cancer is a genetic disease caused by stochastic accumulation of changes in gene sequence, regulation and expression. Notwithstanding, to take breast cancer as an

example, the major gene signatures are defined, with the exception of *HER2*, by expression or absence of expression of genes that are expressed by normal breast epithelia. This observation illustrates that cancers are and should be defined not only by causative genetic and epigenetic events, but also by the extent to which they retain normal gene expression. Adenocarcinomas arise from glandular tissues and the malignant cells are characterized largely by their similarity to the progenitor cells. Appreciation of this phenotype provides insight into their cancer cell biology. Beatson made his breakthrough because he extrapolated from endocrine effects of the ovary on the normal mamma [1].

Trefoil proteins are expressed by all glandular, mucus-secreting epithelia. The questions are: is this normal expression retained by malignant cells and if it is, what are the consequences? Are trefoil proteins mutated in many human tumors? The answer is probably no. Are trefoil proteins important in human tumors? The answer is probably yes. If we understand how they are important then we can ask intelligent questions about their potential as biomarkers. The value of trefoil proteins as biomarkers in human neoplasias cannot be realized in the absence of an appreciation of their biology.

Normal biology of trefoil proteins

The human trefoil factor family comprises three small secreted proteins that are expressed almost exclusively by mucus-secreting epithelia [2,3]. Different epithelia are characterized by secretion of usually one and at most two members of the trefoil factor family. For instance, TFF1 is expressed by mucus-secreting cells that line the stomach, TFF2 by the more basal gastric epithelial

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cells and Brunner's glands of the duodenum, and TFF3 by the goblet cells of the intestines.

Trefoil proteins are defined by the presence of a 42–43-amino acid residue trefoil domain that forms a unique structural motif. Mature secreted TFF2 has 106 amino acid residues and contains two trefoil domains. Secreted TFF1 and TFF3 contain 60 and 59 amino acid residues, respectively, and have a single trefoil domain. A seventh cysteine residue outside the trefoil domain forms intermolecular disulfide bonds and facilitates the formation of TFF1 and TFF3 homodimers [4]. A TFF1 heterodimer of approximately 67 kDa is secreted by breast cancer cells [5]. The major molecular form of TFF1 in gastric mucosa is a 25 kDa heterodimer in which TFF1 is bound covalently to TFIZ1/GKN2, which is an 18.3 kDa secreted protein of unknown function [6].

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The normal function of trefoil proteins is thought to be maintenance of the integrity of and repair of mucosal surfaces. They achieve this by inhibition of cell growth and stimulation of cell motility and invasion, and have been implicated in mucus production, stability and function [7].

Potential role in human cancer

TFF1 was discovered as a consequence of its regulation by estrogen in breast cancer cells [8]. More recently, the induction of TFF3 by estrogen was recognized [9]. Estrogens drive breast cancer progression and their inhibition is clinically beneficial. An important question is, could this therapeutic benefit derive, in part, because trefoil protein expression and secretion is reduced? Many of the functions ascribed to trefoil proteins are suggestive of a promotional role in tumorigenesis. *In vitro* studies that indicate a role in the promotion of angiogenesis have been supported by translational work in gastric [10] and breast cancer [11]. Animal models of, for instance, pancreatic cancer, support a role in metastasis [12], and in breast cancer, TFF1 and TFF3 expression predicts bone metastasis [13]. In apparent contradiction, demonstration that TFF1-null mice develop early gastric cancers [7] and that approximately 50% of human gastric tumors do not express TFF1 [14], suggests that TFF1 might be a tumor suppressor.

Confusion remains in the field regarding the extent to which trefoil protein expression by malignant epithelial cells is ectopic. Initially, it

was thought that TFF1 was not expressed by normal breast epithelia. Similarly, expression of TFF1 in pancreatic adenocarcinoma was wrongly considered to be ectopic. Misconceptions may have arisen due to insensitive assays or because expression may be regulated and hence variable; for instance, in breast epithelial cells during the menstrual cycle [11] and throughout a woman's reproductive life [15]. Malignancies that arise in metaplastic tissue cause confusion. TFF1 and TFF2 are not expressed in squamous epithelium but are expressed in Barrett's metaplasia of the esophagus. Expression of TFF1 and TFF2 in esophageal adenocarcinomas that arise in a background of Barrett's metaplasia should be regarded as retained rather than ectopic, and absence of expression should be regarded as loss of expression. The same argument applies to expression of TFF3 in intestinal-type gastric adenocarcinoma that arises in a background of intestinal metaplasia.

Trefoil factors are considered by some to be beneficial proteins whose loss militates towards cancer [7,16], and by others as detrimental proteins whose gain promotes a more aggressive phenotype [17–19]. This divergence of opinion has divided the field. Recent work in the setting of breast cancer provides support for both camps and a rational resolution of this conundrum [11]. It is clear that TFF3 is expressed in and undergoes polarized secretion from normal lobular and ductal epithelial cells into the lumina. In younger women, the pattern of expression and secretion varies with the menstrual cycle. Retention of expression and reversed polarised secretion of TFF3 from invasive tumor cells is associated with muscle and skin infiltration, lymphovascular metastasis, involvement of lymph nodes and extracapsular spread. Thus it is not that TFF3 changes its function, it is that TFF3 functions in a different milieu.

The future for trefoil proteins as biomarkers

In light of the above, what is the future for trefoil proteins as biomarkers in human cancer as we move into the era of stratified medicine? Five main areas require consideration. The first is a consequence of trefoil factors being secreted proteins; their effect is felt beyond their cell of origin. Therein lies the crux of the question; which cells are being stimulated by the proteins? In normal breast epithelia and in well-differentiated areas of tumors, TFF3 is secreted into the lumina and exerts its normal protective influence on the epithelial mucosal layer. In invasive tumors or in areas of invasion, secretion

is into the stroma, which posits TFF3 in the vicinity of adjacent malignant and stromal cells. In this situation, TFF3 may have a more malign influence, to drive tumor cells down the metastatic cascade by repression of cell growth and death and promotion of invasion both directly on the malignant cells and indirectly on the vasculature. The question is not only do the malignant cells express the proteins, but also to where are the proteins being secreted? Put another way, if trefoil proteins are being secreted by normal or quasi-normal epithelial cells into ductal lumina, this is indicative of good differentiation and prognosis, but if they are being secreted by invasive malignant cells into the stroma then this is indicative of a metastatic phenotype. For example, expression and secretion of TFF1 from the gastric mucosa into the gastric lumen is normal and beneficial, but secretion of TFF1 by malignant gastric epithelial cells into the stroma is bad news.

The second consideration is whether different molecular forms of trefoil proteins should be discriminated. Available evidence suggests that they should. *In vitro* studies have shown that the TFF1 dimer has greater potency than the monomer [20]. Translational studies demonstrate that gastric tumors that express TFF1 are more likely to metastasize if the heterodimer is absent [21].

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The third area is whether or not TFF1 and TFF3 are predictive markers of response to endocrine therapy. There is substantive evidence in support of TFF1 [22,23]. The jury has not been but should be convened for TFF3. Likewise, their ability to predict response to hormonal therapy should be investigated in other tumor types [24].

The fourth aspect is the relationship of trefoil proteins to metastasis. TFF3 discriminates between more or less metastatic cells; higher

expression was associated with propensity to invade the lymphovascularity or the axilla. Notably, TFF3 expression discriminated grade 3 breast tumors [11]. Consideration should be given to the possibility that trefoil proteins are prognostic biomarkers whose measurement could supplement nodal status and identify low-histological grade tumors with worse prognosis or high-histological grade tumors with better prognosis [11].

The fifth area is how intratumor heterogeneity affects the value of trefoil proteins as biomarkers. Presumably for prognostic biomarkers of invasion and metastasis, expression by even a minority of cells will be of import. For predictive biomarkers, it is critical to decipher the proportion of cells that will be targeted successfully. A patient might achieve little long-term benefit from endocrine therapy if only 10% of the tumor cells respond. However, the effect of secreted proteins is felt *a priori* beyond the originator cell. If those 10% of responsive tumor cells express and secrete proteins known to promote invasive phenotypes, then targeted elimination of those tumor cells may be clinically significant. Trefoil proteins may have value as predictive biomarkers both of responsiveness and of the potential worth of endocrine therapy.

In conclusion, the majority of biomarker studies are predicated on simple linear associations between total RNA or protein levels and clinical parameters. Trefoil proteins are a textbook example of how a nuanced approach in which analysis of expression of different molecular forms of the protein in the context of tumor architecture is required.

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