

Diagnostic Potential of Genomic and Proteomic Signatures in Oral Cancer

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ABSTRACT Oral cancer is a public health problem with increasing incidence and mortality rates worldwide. Despite rapid advances in treatment, 5year survival rates have not improved significantly. Major thrust is being laid on diagnosis of the disease in early stages, which is hampered by non-availability of specific diagnostic markers. Advances in genomics and proteomics have made global assessment of expressed genes and proteins in clinical samples feasible. Gene and protein expression profiles derived from clinical specimens have been used to distinguish differences between normal and malignant oral tissues, which are not obvious by clinical, or histologic characteristics. This review focusses on comprehensive analyses of gene expression patterns and proteomic signatures of oral dysplastic lesions and squamous cell carcinomas that have shown considerable promise to improve the discovery of biomarkers for progression of premalignant lesions, prediction of clinical outcome, identification of novel targets for therapy and future directions of molecular signatures of oral cancer in disease diagnosis and therapy.

INTRODUCTION

Oral Cancer is a major global public health problem with 300,000 new cases diagnosed annually. It is the most common cancer in males and third commonest cancer in females in India. Despite rapid advances in multimodality therapy, the morbidity and mortality rates of this devastating disease have not improved in decades. Early detection of oral cancer is the most effective way to improve survival. The treatment planning of oral cancer is mainly based on the tumor, node and metastasis (TNM) classification and histopathological diagnosis. These methods are subjective and often lack sensitivity to detect the disease in early stages. Furthermore, these methods do not reflect the aggressiveness of tumors, prognosis and response to therapy. Therefore, there is an urgent need to develop biomarkers to: identify high risk individuals, improve cancer detection in early stages, predict disease outcome and response to therapy. Rapid advances in high throughput genomic and proteomic technologies have paved the way for better understanding the molecular pathogenesis of oral cancer and identify candidate biomarkers for oral cancer. This review will focus on ongoing

research on genomic and proteomic signatures of oral cancer, our current understanding of molecular basis of oral carcinogenesis, characterization and validation of predictive / diagnostic potential of candidate biomarkers identified from human oral squamous cell carcinoma (OSCC) tissues as well as body fluids such as serum and saliva from oral cancer patients.

MULTISTEP ORAL CARCINOGENESIS

The development of oral cancer is a tobacco related multistep and multifocal process involving field cancerization and intraepithelial, clonal spread. Oral leukoplakia is the most common oral premalignant lesion (OPL) and is often a precursor of oral squamous cell carcinoma (OSCC) (Scully and Porter 2000 a, b). Erythroplakia, oral lichen planus and oral submucous fibrosis constitute other OPLs and premalignant conditions with well defined risk of malignant transformation. Histopathologically, OSCC development is a progress through a series of stages, from hyperplasia to varying degrees of dysplasia and to carcinoma *in situ*, prior to the development of invasive squamous cell carcinoma (reviewed in Reibel 2003; Hunter et al. 2005). The average annual rate of malignant transformation of oral leukoplakia has been proposed to be 1% based on several studies (Hunter et al. 2005). However, a recent study on long term treatment outcome of OPLs showed 7

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fold higher risk of non-homogenous leukoplakia for malignant development as compared with homogenous leukoplakia (Holmstrup et al. 2006). The “field cancerization” theory proposes that an area of oral mucosa can sustain an initial injury from repeated carcinogen exposure and proliferate in a premalignant state. Accumulation of additional genetic insults results in individual subsites of the premalignant field and causes progression to frank carcinoma (Ha and Califano 2003). According to this theory, relatively large areas of mucosa may have a high propensity of eventually developing carcinomas, yet not exhibit all the classic histological markers of malignancy.

Molecular signatures are the qualitative and quantitative patterns of groups of biomolecules (mRNA, proteins, peptide or metabolites) in a cell, tissue, biological fluid, or an organism. To apply this concept to oral cancer biomarker discovery, the measurements should ideally be non-invasive and performed in a single readout. The genetic characterization of field cancerization by Braakhuis et al. (2005) proposes the following model for head and neck carcinogenesis. A stem cell located in the basal layer of the oral mucosa acquires a genetic alteration in the initial phase. The division of this altered stem cell into daughter cells gives rise to a clonal unit of cells that all share this DNA alteration and constitute a patch. Subsequently, accumulation of additional genetic alterations result in the change of a patch into an expanding field that pushes the normal epithelium aside. These fields though macroscopically invisible may appear as oral patches. Finally, clonal selection within this field of preneoplastic cells leads to the development of cancer.

Molecular Alterations in Oral Cancer

Several epigenetic, genetic and metabolic alterations resulting from exposure of oral mucosa to tobacco carcinogens, alcohol or human papilloma virus have been identified in the multistep process of oral carcinogenesis. These have been extensively reviewed by us and others and thus will not be described here (Kaur and Ralhan 2003; Reibel 2003; Nagpal and Das 2003; Schliephake 2003; Bettendorf et al. 2004; Chimenos-Kustner et al. 2004; Warnakulasuriya and Ralhan 2006).

Comparative genomic hybridization (CGH), fluorescence *in situ* hybridization (FISH) and cytogenetic techniques have been used to detect

chromosomal rearrangements and genomic instability in OSCCs (Baldwin et al. 2005; Snijders et al. 2005). Extensive studies have reported the clinical significance of tumor suppressor genes and oncogenes in smoking associated OSCC in South East Asian populations including India. Some of these studies underscore the need to carry out multicentric studies in different populations with well defined tobacco habits for effective translation of biomarkers from bench to the clinic in different population and not translate the panel of biomarkers identified in Caucasians to the Asian population without large scale validations. Though several genes listed above have been associated with clinical and histopathological staging of OSCC (p16, p53, cyclin D1, p14^{ARF}, Ets-1), these markers provide little predictive or prognostic information for translation into a clinical setting (Arora et al. 2005a; Soni et al. 2005). In view of the vast heterogeneity in OSCCs it is unlikely that a single biomarker will be useful for early detection of all OSCCs. Therefore, there is a need for identification of a panel of biomarkers for diagnosis of OSCC.

Genomics and Proteomics for Biomarker Discovery in Oral Cancer

The advent of array technologies have provided a high throughput approach to monitor RNA for mRNA expression and DNA for genomic polymorphisms. In addition, epigenetic changes such as global hypomethylation and promoter hypermethylation of CpG rich areas have also been investigated. Rapid advances in proteomic technologies and mass spectrometry have provided the tools for analysis of protein profiles of OPLs and OSCCs. Furthermore, major thrust is being laid on analysis of DNA, RNA and protein profiles of biological fluids such as serum, plasma, saliva and urine for identification of biomarkers using non-invasive methods for multiple analyses, not only for early detection of oral cancer, but also for monitoring prognosis of the disease and response to therapy. Studies carried out using these techniques confirm the involvement of multiple pathways and cross-talks between pathways in oral tumorigenesis.

Genomics Signatures of OSCC

DNA microarrays are powerful tools for measuring gene expression profiles on a tissue-

wide level. Microarrays consist of millions of individual nucleotide sequences affixed to a solid substrate in a defined configuration. These nucleotide sequences (called “probes”) are complementary to the sequences of known and putative genes. Messenger RNA (termed “target”) that is extracted from a tissue of interest can be labeled with a fluorescent tag and hybridized to the microarray. By measuring the intensity of fluorescence at the precise locations of each probe on the microarray, the abundance of the target mRNA can be determined. The highly parallel nature of microarray technologies allows for the simultaneous measurement of thousands of genes in a relatively rapid manner. A variety of experimental platforms, probes, hybridization and signal detection methods, computational and bioinformatic analyses programs are being developed for global gene expression profiling both at micro- and macro- levels. Our group has previously identified differentially expressed gene patterns between OSCC and normal oral mucosa (Arora et al. 2005b). DNA microarrays have extensively been used to compare gene expression profiles of OSCCs with normal healthy oral tissues and reviewed (Todd and Wong 2002; Kuo et al. 2003; Otao-Rey et al. 2004; Brigitta and Wong 2006). These studies have led to identification of clusters of genes (also known as molecular signatures) that are associated with OSCC, thereby providing insight into the molecular mechanisms involved in oral tumorigenesis and identify candidate novel tumor derived biomarkers (Alevizos et al. 2001; Mendez et al. 2002; Conrads et al. 2003; Hwang et al. 2003; Chimenos-Kustner et al. 2004; Diamandis 2004; Carinci et al. 2005; Cheng et al. 2005; Dallas et al. 2005; Drake et al. 2005). Strikingly, there is a vast heterogeneity in the findings reported in several different studies. Kuo et al. (2002 a) suggested the possibility of classifying OSCC on the basis of gene expression patterns. Using a microarray of 4000 genes, they identified 210 genes that may be related to oral cancer. Some of these genes (CKS1, TSPY, CBK, TLEY and BCHE) have previously been reported in other cancers, but not in oral cancer. In addition, these authors also listed a set of genes whose expression is correlated with other classic prognostic factors, such as p53, MST1, HLA-DBQ1 and UBA52. Alevizos et al. (2001) and Mendez et al. (2002) also provided verifiable molecular signatures of

OSCC using DNA microarrays ; these data have been reviewed by Otao-Rey et al. (2004).

Another interesting study by Kuo et al. (2002 b) comparing gene expression profiles of oral normal, dysplastic and malignant cells using DNA microarrays and laser capture micro dissection found several genes and expressed sequence tags (ESTs) to be associated with cancer namely DDB2, a damage-specific DNA protein, to be a potential biomarker for the progression of OSCC. Intriguingly, expression profiles of dysplasia samples did not cluster together as a group, but were found in either ‘normal’ or ‘cancer’ clusters, suggesting that subtle changes occur in the progression of OSCC that are not captured by histopathology.

Odanti et al. (2006) compared gene expression profiles of oral leukoplakia and OSCC using Affymetrix Gene Chip system and suggested that gene abnormalities in cytoskeleton network components might be responsible for the development and progression of oral leukoplakia. Though the small sample size (4 leukoplakia and 2 OSCC) is a major limitation of this study. Nevertheless, it demonstrated that some of the 18 alternatively expressed genes were markedly down-regulated in OSCC compared with leukoplakia, further confirming the complexity of molecular alterations in the progression of OSCC proposed by the earlier study of Kuo et al. (2002 b, 2003a).

A major limitation of most studies using microarrays is that known OSCCs have been analysed and compared with normal counterparts. The major challenge is to identify molecular signatures that can accurately predict OSCC in unknown tissue specimens. In this context, Whipple et al. (2004) showed that principal component analysis (PCA) can be used with genomic microarray data to correctly predict the presence of OSCC in unknown tissue samples. Leethankul and co-workers (2000) have identified several differentiation and growth related genes in HNSCC using normal and malignant keratinocytes obtained from biopsy specimens by LCM using DNA microarray containing 588 cancer related genes . Oral cancer molecular signatures have also been investigated using high density oligonucleotide arrays. Ohyama et al. (2000) demonstrated the feasibility of procuring high amounts of target sample using LCM generated tissues for hybridizing high density oligo arrays. Using this optimized technology Alevizos et al. (2001), Mendez et al.

(2002) and others carried out gene expression profiling of OSCCs.

Giri et al. (2006) determined the molecular signatures associated with clinical outcome in patients with high risk head and neck cancer squamous cell carcinoma (HNSCC) treated with surgery and radiation. Patients who developed distant metastases has 205 genes differentially expressed as compared to patients with no recurrence. Genes associated with cell growth and proliferation; cell adhesion, DNA replication, recombination and repair, antiapoptotic pathways and angiogenesis were identified in tumors of patients with distant metastases. In contrast, tumors of patients with no recurrences showed discriminatory genes associated with the onset of apoptosis.

Liu et al. (2006) used array-CGH to detect genome wide changes in microdissected primary and metastatic OSCCs. Genomic alterations (TGFB2, cellular retinal binding protein 1 gene (CRBP1), PIK3CA, HTR1B, HRAS, ERBB3 and STK6) differed significantly between primary OSCC and neck lymph node metastatic tissues. An intriguing finding was the significant difference in PRKCZ, ABL1 and FGF4 in patients who died compared with those who survived. These findings after validation in larger cohorts could provide a panel of biomarkers for prognosis of OSCC.

Using array CGH O'Regan et al. (2006) demonstrated less genomic instability in young nonsmokers with OSCC than found in typical patients (smokers) with oral cancer. Bremmer et al. (2005) developed a non-invasive genetic screening test for detection of OPLs based on multiplex ligation-dependent probe amplification (MLPA). MLPA enabled measurement of gains and losses at 40 different chromosomal locations in a single PCR reaction using only 150ng DNA obtained by brushing the oral mucosa. Validation of this method on large sample size could be a promising method for screening of OPLs. Transcriptional profiling in OPLs is crucial for revealing molecular and biological changes resulting from oral epithelial transformation. Some studies such as the reports of Banerjee et al. (2003, 2005) highlighted dysregulation of several genes related to arachidonic acid metabolism and related inflammatory cascades suggesting that this pathway may be involved in the irreversible transition to progressive stages of oral cancer. While the other studies by Warner et al. (2004)

identified distinct patterns of gene expression associated with clinical parameters in OSCCs.

Irie' T et al. (2004) showed that genes which are important for histogenesis and acquisition of invasion and proliferation capability are commonly aberrant in OSCCs. Noteworthy studies by Roepman et al. (2005) and O'Donnell et al. (2005) independently identified and validated primary tumor tissue expression signatures that can detect the presence of local lymph node metastasis reliably. In contrast, in the recent study by Braakhuis et al. (2006) no specific metastasis signature was identified in HNSCC including OSCC. These conflicting reports may reflect heterogeneity of HNSCC with respect to metastatic behavior or may be attributed to the relatively small patient groups used in this study. In conclusion though signatures of differentially expressed genes have been identified it is perhaps too early to state whether a useful signature exists. The analysis of much larger numbers of HNSCCs will be required to validate if a specific molecular signature exists for distant metastasis. DNA hybridization arrays have been used for identifying novel gene clusters predictive of radiation responsiveness of OSCC (Hanna et al. 2001). Using DNA microarrays, Higo et al. (2005) identified a panel of 7 candidate radioresistant genes (CK18, DTNBP1, ASNA1, Tep20, Cyclophilin F, K1Aa0218 and HBp17) that could be useful in identifying radioresistant OSCCs.

Oncogene expressing human papilloma virus type 16 (HPV16) is found in a subset of HNSCC including OSCC. Smeets et al. (2006) used microarray CGH to compare genome wide DNA copy number alterations in HNSCC with or without HPV. Four regions (losses at 3p11.2-26.3, 5q11.2-35.2, and 9p21.1-2y and gain at 11q12.1-13.4) showed alterations in HPV negative tumors that were absent in HPV positive tumors. However, 7 regions were altered at high frequency (>33%) in both groups. This study suggests that HNSCC including OSCC arising by environmental carcinogens are characterized by genetic alterations that differ from those observed in HPV-16 induced tumors and most likely occur in early stages of carcinogenesis, while numerous genetic changes that are shared in both tumor groups may be considered crucial in the later stages of tumor progression.

Intense efforts are being directed towards analysis of DNA and RNA in body fluids such as

serum, plasma, saliva or urine, using array technologies. Human DNA biomarkers have been identified in saliva and used for oral cancer detection (Liao et al. 2000; El Naggar et al. 2001). An excellent example is the noteworthy study by Li et al. (2004) on salivary transcriptome analysis for oral cancer detection. Microarray analysis showed that 1679 genes exhibited significantly different expression level in saliva between OSCC patients and controls. Seven salivary RNAs combined were able to predict the presence of oral cancer with 91% specificity and sensitivity. These potential salivary RNA biomarkers are transcripts of IL8, IL1B, DUS, HA3, OAZ1, S100P and SAT. The utility of salivary transcriptome diagnostics for oral cancer detection as a non invasive tool with sufficient predictive power has been demonstrated in this study. This novel clinical approach could be exploited as a high throughput tool for early cancer detection if validated in other independent studies. Some of these have been confirmed in a later study in 272 OSCC patients (Brinkman and Wong 2006).

High density oligonucleotide microarrays have recently been used for the first time by Li et al. (2006) for global transcriptome profiling from serum for OSCC detection. Human serum circulating mRNAs were amplified by RTPCR and microarray identified 335 serum RNAs to be significantly differentially expressed in OSCC patients as compared to the controls. Five cancer related gene transcripts (H3F3A, TPT1, FTH1, NCOA4 and ARCR) were validated giving a sensitivity of 91% and specificity of 71% in distinguishing OSCC.

These studies illustrate that expression profiling using microarrays is a means to refine conventional assessment of OSCCs allowing for a more accurate prediction of disease prognosis. There is a need for integrated microarray database the would allow comparison of microarray data generated from different laboratories. Molecular signatures of OSCC will ultimate translate into early detection, more predictable response to therapy and development of novel molecular therapeutics.

The ultimate goal of oral cancer proteomics is to adapt proteomic technologies for routine use in clinical laboratories for diagnostic and prognostic use, as well as in evaluating response to therapy including drug toxicity and efficacy. In addition, proteome analysis also enables evaluation of regulation of protein function by

proteolysis, recycling and isolation of subcellular compartments that effect gene products. Finally, protein-protein interactions and the molecular composition of cellular structures can also be determined only at the protein level. However, the dynamic nature of the proteome and the immense biological variability among the patient samples are major challenges for deducing diagnostic patterns unique to OSCC. The current biomarker identification strategies for HNSCC, different combinations of MS platforms, LCM and 2D gel electrophoresis procedures applied to readily available clinical specimens (tissue, blood and saliva) have been recently reviewed by Drake et al. (2005). These authors have also discussed issues related to assay reproducibility, management of large data sets and future improvements in clinical proteomics. The same considerations hold for OSCC, therefore these issues will not be further addressed in this review.

Proteomics Signatures in Oral Cancer

In comparison with gene expression profiling of OSCCs using microarrays, the studies on proteome analysis of oral cancer are limiting. The first proteomic analysis of oral tongue carcinoma was reported by He et al. (2004). Protein expression profiles of 10 tumors and their matched normal mucosa resection margins were analyzed by two dimensional gel electrophoresis and matrix-assisted laser desorption / ionization-time of flight mass spectrometry (MALDI-MS). Several tumor associated proteins including HSP60, HSP27, alpha-B-crystalline, ATP synthase beta, calgranulin B, myosin, tropomyosin and galectin 1 were found to be significantly altered in tongue carcinoma tissues compared with their normal mucosa, providing a proteomic signature of tongue carcinoma. Using the same technique, his laboratory also provided the proteomic signatures of buccal squamous cell carcinoma (Chen et al. 2004). These authors observed increased expression of glycolytic enzymes, heat shock proteins, tumor antigens, cytoskeletal proteins, enzymes involved in detoxification and anti-oxidation systems, and proteins involved in mitochondrial and intracellular signaling pathways. These findings indicate that multiple cellular pathways are involved in oral tumorigenesis. Hence multiple proteins need to be simultaneously targeted for rational design of diagnostic and therapeutic strategies. The panel

of candidates for targeted proteins included SCC antigen, G protein, glutathione S-transferase, manganese superoxide dismutase, annexins, voltage dependent anion channel, cyclophilin A, stratifin and galectin 7, although no specific biomarker for buccal SCC was found in this study.

To gain insight into the molecular mechanisms involved in oral carcinogenesis Koike et al. (2005) used fluorescent two dimensional differences in gel electrophoresis and MALDI TOF-MS for proteomic profiling of human normal oral keratinocytes and OSCC derived cell lines. Nine proteins were up-regulated and 13 were down-regulated in OSCC cells. These included annexin A1, HSP27, laminin A/C, interleukin/ receptor antagonist, serine proteinase inhibitor clade B5, stathmin 1 and superoxide dismutase 2. Maddola et al. (2005) have developed MALDI Imaging MS proteome analysis technique to be used on thin histological samples and a specific statistical method of analysis to enable data processing in the absence of internal standards by defining similarity scores. However utilization of this technique in clinical specimens remains to be tested.

Differentially expressed metastasis-associated proteins in adenoid cystic carcinoma cell lines of human salivary gland have been identified using 2D-MS. Some of the proteins associated with tumor metastasis included transketolase, Dim1p, v-Ha-ras oncogene, type I collagen protein alpha, tumor necrosis factor (ligand) superfamily member 4 and pirin. However, the functions of these proteins in tumor metastasis remain to be determined (An et al., 2004).

Nakashima et al. (2006) identified maspin and stathmin as potential biomarkers and also markers of biological behaviour of adenoid cystic carcinoma of the salivary glands using fluorescent-2-dimensional differential in gel electrophoresis (2D-DIGE)-MALDI-TOF-MS. 2D-DIGE is an emerging technique for comparative proteomics that improves the reproducibility and reliability of differential protein expression analysis between the samples. Fluorescent 2-D differential in-gel electrophoresis (2-D-DIGE) allows multiplex analysis of 3 sample proteomes on the same gel. (Tonge et al. 2001; Skynner et al. 2002; Zhou et al. 2002). The protein extracts being compared are covalently labeled with different fluorescent Cy Dyes, which are N-hydroxysuccinimidyl ester derivatives of Cy2, Cy3 and Cy5. Fluorescent imaging of the gel at the wavelengths specific to each Cy Dye generates images that can be

overlaid directly by the DeCyder software to identify any differentially expressed proteins between the samples.

Cheng et al. (2005) developed a bead based affinity fractionated proteomic method to discover a novel plasma marker for oral cancer. Affinity purification of heparinized plasma with copper chelated beads and MALDI-TOFMS analysis were used to screen potential oral cancer markers from 57 OSCC patients and 29 healthy controls. The spectra were analysed using flex analysis™ and Clin-Prot™ bioinformatic softwares. Six markers that differentiated between cancer and control spectra were found with mean molecular masses of 2664, 2850, 3250, 7735, 7927 and 9240 Da. The marker identified as a fragment of the fibrinogen a-chain had the highest sensitivity (100%) and specificity (97%) for cancer, suggesting that it may be a clinically useful tumor marker. However, clinical studies on a larger sample size are needed to determine whether this biomarker can be used for diagnostic purposes.

Affinity bead-based purification has been developed to reduce costs and make proteomic procedures suitable for general MS analysis. This method uses different chemical chromatographic surfaces on an outer layer of magnetic beads to selectively purify certain subsets of proteins, allowing unbound impurities to be removed by washing with buffers. Protein bound to the magnetic beads are then eluted, diluted, and directly analyzed by MALDI-TOF MS.

Serum proteomics and peptidomics are gaining popularity among oral oncologists in their quest for cancer biomarkers with high diagnostic accuracy. This is based on the premise that sera from oral cancer patients contain small proteins and peptides detectable by mass spectrometry (MS) that reflects the presence and biology of a tumor.

There are very limited studies comparing transcriptome and proteome analysis. In this context the noteworthy study by Roesch Ely and coworkers (2005) combined cDNA microarrays, quantitative reverse transcriptase-PCR (qRT-PCR) and surface enhanced laser desorption ionization (SELDI)-TOF MS to study differential gene expression in HNSCC. Calgranulins A and B and Annexins 1 and 2 were found to be down-regulated in HNSCC compared with normal mucosa, at both the mRNA and protein level, and these findings were confirmed by validation in HNSCCs. Hypoxia decreases therapeutic

efficacy in solid tumors. Therefore identification of hypoxia markers may influence the choice of therapeutic modality. With this rationale Chen et al. (2004) using 2 D-gel electrophoresis and Power Blot (antibody- based array), identified a group of 20 proteins upregulated during hypoxia. These proteins included I Kappa B kinase beta (IKK beta), MKK3b, highly expressed in cancer (HEC), density regulated protein 1, P150 (glued), nuclear transport factor 2, binder of ARL 2, Poxillin, and transcription termination factor 1. Correlation of IKK beta expression in HNSCC specimens with tumor oxygenation suggested that IKK beta may be a novel endogenous marker of tumor hypoxia and may represent a novel target for therapy. Melle et al. (2004) using a technical tirade of tissue microdissection (LCM), protein Chip arrays and immunohistochemistry for proteomic identification of HNSCC showed calgranulin A (S100A8) and calgranulin B (S100A9) to be candidate biomarkers for HNSCC. Wu et al. (2002) identified metastasis associated proteins in HNSCC cell lines using SELDI Protein chip technology – MS. Enolase-alpha, annexin I and annexin II were found to be upregulated and may be important in head and neck cancer invasion and metastasis.

Clinical Validation of Biomarkers

Despite of large amount of data obtained from several expression profiling studies worldwide, there is a gap between the knowledge accrued in the laboratory and its translation into clinical practice. Validation of the data poses a major challenge. Most studies have been carried out on a small sample sizes and often involved cancers from different anatomic sites. Therefore, there is a need for large scale validation in prospective, well designed clinical studies in multicentric settings. The Early Detection Research Network by National Cancer Institute aims to rapidly bring molecular markers to the clinic. Similar initiatives by other countries would considerably aid the quest for validation of candidate novel biomarkers for translation from bench to bedside.

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