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BIOMARKERS AND THE GENETICS OF EARLY NEOPLASTIC LESIONS

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1. INTRODUCTION

Two great challenges in cancer diagnosis and prevention are 1) the detection of specific pre-invasive neoplastic lesions that give origin to malignant tumors and 2) the identification of those prognostic factors of tumors that predict the outcome of individual cancer patients. As personalized medicine becomes more established, the use of biomarkers to predict responses to various therapeutic regimens also will become much more important. Also, the ability to detect neoplastic transformation at its earliest presence is a requisite for improving preventive interventions and reducing cancer incidence.

In this chapter we will discuss the molecular aspects of early pre-invasive neoplastic lesions; specifically, alterations in genes regulating proliferation, differentiation, apoptosis and invasiveness as well as chromosomal aberrations and microsatellite instability will be addressed. We will argue that the identification of molecular changes associated with neoplastic transformation will lead to the development of new molecular markers for the early detection of pre-invasive neoplastic and malignant lesions, assessment of cancer risk, assessment of responses to preventive or therapeutic interventions, and separation of less aggressive neoplastic lesions from more aggressive lesions.

1.1. Early Neoplastic Lesions: Definition and General Concepts

Neoplastic lesions may derive from epithelial or nonepithelial cellular populations. Often, pre-invasive neoplastic lesions are small, multiple and may display wide ranges of diversity [1]. Morphological alterations characterizing pre-invasive neoplastic lesions include nuclear pleomorphism consisting of increased variation in nuclear size, shape, and hyperchromatism, abnormal mitoses, abnormal nucleolar patterns and altered or absent differentiation. The evolution of these types of lesions may take three directions: spontaneous regression, progression toward a fully malignant phenotype, or stasis, remaining as a pre-invasive neoplastic lesion (2). Although there are discrepancies in terminology used by pathologists to describe these types of early neoplastic lesions, they are most frequently referred to as pre-invasive neoplasia, dysplasia, carcinoma *in situ*, intraepithelial neoplasia, or as incipient cancer (3,4).

Based exclusively on morphological features, lesions which progress to malignant tumors cannot be easily distinguished from lesions which spontaneously regress. It has been shown

that normal epithelium and normal-looking tissue in the proximity of a tumor may carry mutated cancer genes, suggesting that the first steps in early pre-invasive neoplasia do not have a distinctive morphologic correlate (5–8). Similarly, molecular changes in lesions known to be at risk for developing morphologic pre-invasive neoplasia such as ulcerative colitis, actinic skin damage, and Barrett's esophagus, may precede histomorphologic changes by many months (9,10). Development of molecular markers, which clearly reflect various stages of neoplastic transformation, may redefine the diagnostic criteria for precursor lesions of cancer (11).

Carcinogenesis is an indivisible continuum of molecular and morphological changes that may culminate in the development of invasive tumors. A growing body of evidence suggests that a tumor may initially start as a stem cell that develops mutations and subsequently produces a clonal population of cells harboring these and additional mutations which uniquely suit the clone to its microenvironment. Because early neoplastic lesions exhibit phenotypic heterogeneity, multicentricity, genetic instability and other chromosomal alterations, it is not always easy to predict their biological behavior or risk of progression [12]. Studies in which the molecular features of pre-invasive neoplastic lesions are correlated with molecular features of matching invasive lesions, for example, a colorectal cancer with an adenomatous polyp forming its edge, should be useful in identifying molecular features associated with greater risks of progression (13). Similarly, studies of prognostic factors in specific cancers may identify molecular features associated with aggressive behavior (14). Good transgenic mouse models of a cancer (e.g., pancreatic cancer) also may be useful in identifying molecular features leading to the development of metastatic disease (15,16). In some cases it is the combination of molecular changes, not one molecular change, that leads to aggressive behavior. For example, in mouse models mutations in K-ras frequently lead only to pancreatic intraepithelial neoplasia (PanIN) and only loss of p16/p19 (Ink4a/Arf) may not cause major changes in transgenic mice; however, activation of K-ras-2 by mutation plus loss of Ink4a/Arf leads to metastatic pancreatic cancer (16) (See Figure 1). Of note is that some molecular features in one tumor may identify an aggressive subset of tumors, but these same markers e.g. p53, may not identify aggressive features in a different tumor (13,17). The study of early neoplastic lesions is further complicated by observations that cells carrying chromosomal and genetic alterations may disappear during the spontaneous regression of intraepithelial neoplasia, as reported for cervical, skin and bronchial lesions (1,2,17).

Thus it is apparent that molecular changes precede histological and morphological alterations and these findings may have implications for developing a new classification of early neoplastic lesions, based on molecular parameters (11); however, unless there are grossly observable histopathologic changes (e.g., increased vascularity or inflammation), that mark areas in which molecular changes are likely, molecular changes alone are unlikely to be of practical use in identifying the boundaries of pre-invasive neoplastic lesions because the pattern of sampling would be too complex and the many assays to demark the boundary of the lesion would be too costly.

1.3. Molecular Basis of Early Neoplastic Lesions

Because a proportion of high grade pre-invasive neoplastic lesions progress to frank cancers, pre-invasive neoplastic lesions must develop part, but not all of the features of the associated cancers. The six “hallmarks of cancer” as proposed by Hanaken and Weinberg (18) also must apply in part to pre-invasive neoplasia. Actually, an additional hallmark of cancer not listed by Hanaken and Weinberg should be the ability of neoplastic lesions to evade partially or completely immune surveillance. Thus, the most important hallmarks of pre-invasive lesions are:

- 1) Self sufficiency in growth signals
- 2) Evasion of apoptosis
- 3) Insensitivity to anti-growth signals
- 4) Evasion of immune surveillance
- 5) Limitless replicative potential

Because pre-invasive neoplastic lesions are in situ and some of these lesions regress, the following hallmarks of cancer are less likely to be important to the maintenance of pre-invasive neoplasia.

- 6) Sustained angiogenesis
- 7) Capability for tissue invasion and metastasis

Molecular alterations involved in human carcinogenesis are very diverse, as are the mechanisms by which cellular functions may be altered (4,19,20) (Table 2). It has been implied that an increased rate of mutation could be the most important factor in tumorigenesis although this assumption is not universally accepted (21–24). In any case, mutations in genes controlling DNA repair and DNA synthesis appear to compromise the genetic stability of cells and contribute to the progression of neoplastic changes, which, in turn, leads to the increase of genetic alterations in emerging clones. Mutations that provide a selective growth advantage appear to play a key role for carcinogenesis (25). Based on the key factors that lead to cancer, early neoplastic transformation would rely on molecular changes that avoid apoptosis and facilitate continued proliferation by not responding to factors that inhibit growth and developing combinations of factors that self-sufficiently stimulate growth that is not limited in its replicative potential. Unlimited growth is supported via the maintenance of the lengths of telomeres by the action of telomerase which is controlled by multiple trans-activating transcriptional regulators such as myc (18,21). For example, a setting of long standing continuing damage, inflammation and repair (LOCDIR) which occurs in conditions such as ulcerative colitis, puts pressure on stem cells to proliferate (4). This proliferation occurs in a setting in which factors are present (e.g., reactive oxygen and reactive nitrogen species) that inhibit complete repair which may lead to genetic damage to stem cells. When such genetic changes develop in a specific stem cell and these changes favor rapid and continuing growth of the specific stem cells in their microenvironment, a pattern of clonal growth may develop. When this clonal growth also develops methods to produce sustained angiogenesis, invasion, and metastasis, a malignant tumor may develop. The clonal progeny of a stem cell may subsequently develop alterations in gene expression that lead to the development of sustained growth (12,18,21,26). It has been postulated that clonal selection continues throughout various stages of tumor growth, which may explain the sequential emergence of altered populations of cells over time (25). For example, such a hypothesis of clonal selection is in agreement with the emergence of multiple pre-invasive neoplastic lesions, and sometimes second primary tumors. This pattern is known as “field effects” that can be induced by carcinogen-exposure, as first described by Slaughter (27). This is discussed further in a subsequent section.

Epigenetic changes, in particular the methylation of DNA, as well as factors in the tumor microenvironment, such as hormones, vitamins, prostaglandins, growth factors and cytokines, play an important role in neoplastic transformation. These factors may markedly influence the evolution of pre-invasive neoplastic cells by accelerating, retarding, or inhibiting their transformation into fully malignant cells, or even reversing their characteristics to a normal phenotype (28). For example, it has been shown that in an organ such as the prostate that circulating cytokines released by underlying stromal cells may modulate normal epithelial differentiation, proliferation, ductal morphogenesis and protein

secretion (29). In turn, secreted factors from prostatic epithelial cells may alter the underlying stroma, which supports the hypothesis that dysregulation of the cross-talk between epithelial and mesenchymal circulating cytokines is involved in carcinogenesis (30).

Another major feature of all malignant tumors is the partial avoidance of the effects of death of the neoplastic cells. Tumor growth is caused by the unequal balance between cellular proliferation and cellular death. In cancers, there may be several types of cellular death, e.g., ischemic necrosis; however, most forms of individual cellular death are sometimes incorrectly combined under the term apoptosis – a death of the cell directed by endogenous causes which affect the cellular DNA. Thus, one of the features required for neoplastic lesions to progress to frankly malignant tumors is described as avoidance of apoptosis (18). Apoptosis is a cellular death induced by an intracellular program attacking DNA and nuclear and cytoplasmic proteins while leaving cellular membranes intact. There are two main pathways leading to apoptosis – one via the extrinsic pathway (death receptor initiated) and one via the intrinsic pathway (mitochondrial associated pathway). There are multiple stimulatory and inhibitory factors that affect either or in some cases, both of these pathways. For example, there are more than 20 proteins in the Bcl-2 family which regulate apoptosis primarily via mitochondrial associated pathways. The two main anti-apoptotic proteins are Bcl-2 and Bcl-X and the three main pro-apoptotic proteins are Bak, Bax and Bim. Thus, apoptosis tends to be a balance of the various stimulatory and inhibitory proteins associated with the pathways of apoptosis. Nevertheless, one has to be very careful in interpreting the effects on differential expression of these molecules on tumors. For example, increased levels of Bcl-2 are associated with a good prognosis in colorectal and breast adenocarcinomas, but with a bad prognosis in prostate, bladder, and hematopoietic malignancies (31–33). Similarly, the p53 mutation has been associated with normal appearing skin (10) and with pre-invasive neoplastic lesions of tissues such as breast, skin and SCC of the oral cavity, while in other types of tissue mutations of or dysregulation of p53 has been associated with advanced or metastatic lesions, e.g., prostate (34).

Recent findings suggest that apoptosis is defective in neoplastic cells and that the failure of cells to die in response to damage may permit the progression of the pre-invasive neoplastic lesion to frank malignancy during the pre-invasive state. Impairment of apoptosis is involved at very earliest stage of neoplasia, as demonstrated in colon epithelial cells harboring a APC mutant gene (35).

The study of oncogenes and tumor suppressor genes has provided critically important information for our understanding of the molecular events underlying the sequence of changes leading to tumor development (36). Mutations, which may induce the activation of oncogenes or inactivation of tumor suppressor genes, may dysregulate cellular proliferation through a variety of mechanisms. Oncogenes and tumor suppressor genes may affect differentiation, apoptosis, signal transduction, intercellular communication and adhesion, initiation of DNA replication and regulation of expression of certain specific genes (36,37). Alterations in other classes of genes, including those regulating the interaction with the extracellular matrix, as well as neoangiogenesis, may enable cells to acquire an invasive and metastatic capacity (38–42). It is clear that molecular manifestations of neoplastic transformation precede detectable morphological changes and that genomic changes associated with cancer may occur in cells long before morphological alterations become apparent.

2. BIOMARKERS OF EARLY NEOPLASTIC LESIONS

2.1. General Concepts

Biomarkers include any morphological, biochemical or genetic alteration by which a physiological or pathological process can be recognized and monitored. Cancer related biomarkers include those markers which are associated with early neoplastic lesions, as well as the markers associated invasive or metastatic tumors (Table 3). Presumably, molecular events in the causal pathway leading to cancer will be identified by a relatively restricted set of markers. A panel of markers reflecting invasion-related molecular changes will aid in characterizing advanced or metastatic cancers. In the following section, we summarize some critical events that occur during the initiation of pre-invasive neoplasia.

In general, the direction of the differentiated expression (increased or decreased) of biomarkers in pre-invasive neoplastic lesions follow that of invasive lesions. Thus, if p53 is mutated in a colorectal polyp from which a colorectal cancer (CRC) arises, the CRC will usually contain the same mutation in p53 (13). Similarly, if there is strong membrane expression of p185^{erbB-2} in ductal carcinoma in situ (DCIS) of the breast, there is usually similar expression in the associated ductal carcinoma. Also, in general, if biomarkers are high in the majority of pre-invasive neoplastic lesions, they will be high in the metastatic lesions of the same type of tumor; however, there are some exceptions such as the expression of TAG72 (B72.3) in prostate cancer (43).

2.2. Chromosomal Aberrations

Common chromosomal aberrations which may be observed in metaphase preparations after treatment of cells with DNA-damaging agents include breaks, deletions, translocations, amplifications, duplications, circularizations and dicentric. Some chromosomal aberrations, such as translocations or deletions tend to be stably transmitted throughout generations of cells. In contrast, other chromosomal aberrations, such as dicentric, are not passed on the next cell generation, which suggests that they represent an unstable event, caused by a recent clastogen exposure. Studies of changes in chromosomes have added in many ways to our understanding of tumor development and have increased the evidence for the importance of cumulative genetic alterations as a major force in tumor evolution (44,45).

2.2.1. Aneuploidy—Aneuploidy is the most common chromosomal aberration associated with premalignant lesions (46–48). Aneuploidy, or the change in copy number of the chromosomes, is often measured as the DNA index, a ratio of the DNA content of a cell to that of a diploid normal cell. A normal DNA index can be seen in cells having considerable gains or losses in various different chromosomes, and indeed allelic loss at a particular site is often accompanied by no cytogenetically detectable changes in the remaining chromosomes. Based on DNA content, a tumor can be diploid but still may have changes in gene copy numbers or allelic imbalances. Cells that have aneuploidy in DNA may have chromosomal numbers below diploid or above diploid. When the number of chromosomes are twice the diploid number, the cell is described as tetraploid. Octoploid is 4 times diploid. Tetraploid and octoploid cells can occur in normal liver, especially in rodents. Aneuploidy as well as point mutations appear to be associated with conditions predisposing to cancer. Aneuploidy may have value as a complement to histological examination in the surveillance of ulcerative colitis patients, who are known to have an increased risk of developing colorectal cancer (49).

2.2.2. Deletions and Translocations—A deletion usually refers to a large loss of a chromosomal region or even of an entire chromosome; it thus may involve the loss of a single copy of each of many contiguous genes. When such a loss occurs, there can also be an

associated reduplication of the remaining chromosomal copy of the lost area or uniparental disomy (50,51). Such acquired uniparental disomy (UPD) usually results in the under estimation of LOH. In UPD, there is initially LOH, thus a section of a chromosome from one parent is lost leaving the same area on the chromosome from the other parent intact. By methods which are not understood, one or more new copies of the area of chromosomal deletion is reproduced from the remaining area of the chromosome that matches the lost chromosomal area. Thus, there will be at least two copies of the originally deleted chromosomal area, but both copies will be from one parent, i.e., the parent whose chromosomal area was not deleted.

Clinically UPD may be important in that genes may be amplified secondary to UPD and polymorphic proteins may be homozygous more than would be expected. Such changes will not easily be detected (50). Of interest, BRCA1/2 associated serous ovarian carcinomas exhibited more genetic instability and more UPD than randomly selected sporadic serous ovarian carcinomas (51) as would be expected because of the genetic instability induced by BRCA1/2.

Alternately, loss of one parental copy of an area of a chromosome can be accomplished by mitotic recombination with the homologous chromosome, again resulting in a retention of cytogenetically normal structure of the chromosome. Therefore cytogenetic analysis can underestimate the extent of genetic losses. It is consequently more instructive to speak of the loss of one parental copy of a gene, of heterozygosity (LOH) or allelic loss. Deletion can also be the mechanism for loss of both copies of a gene (homozygous deletion). This usually combines a large LOH deletion with a much smaller deletion that involves one gene or scores of genes. For example, when one copy of p53 is lost, there is frequently a loss of the other copy, resulting in a homozygous deletion of p53. Because the homozygous deletions of a neoplasm obviously cannot involve any of the genes that are essential for cellular metabolism, replication or survival, their size and prevalence are highly restricted by the types of neighboring genes as well as whether or not there is UPD.

When a LOH involves a major suppressor gene such as p53 or Rb and no other genes critical for cell survival are lost, this genetic change usually results in an increase in cellular functions and/or loss of apoptotic responses. Such changes can lead to neoplasia. Recently, it has been recognized by the Czerniak laboratory (52,53) that in some cases major suppressor genes are surrounded geographically on the chromosome by less critical suppressor genes. A loss of one or more of these minor suppressor genes may occur as an early change of neoplasia; it may stimulate a clonal expansion and may lead to a loss of the major suppressor gene (e.g., Rb). These genes have been designated as “forerunner” genes (52,53). Loss of forerunner genes in the earlier stages of neoplasia followed by the loss of the major associated suppressor gene appears to be common as early neoplastic lesions progress (52,53).

Translocation involves a rearrangement of a portion of one chromosome to another chromosome or a rearrangement within a single chromosome. As a result, the activation of an oncogene may occur. For example, a growth-promoting gene can be placed near a strong or constitutively active transcriptional promoter. Alternately, the introns of two different genes could be united so that the spliced transcripts of the rearranged site produce a fusion of peptide domains, encoding a novel protein (54,55). Perhaps the best known translocation is represented by the Philadelphia chromosome which is present in chronic myelocytic leukemia. It results from translocation of the ABL-1 proto-oncogene positioned on chromosome 9, to a region of chromosome 22 downstream on BCR. This fusion activates the ABL-1 gene resulting in either chronic myeloid leukemia or acute lymphoblastic leukemia, depending upon the chromosomal breakpoint (56). A vast number of

chromosomal alterations associated with hematopoietic cancer, sarcomas and premalignant lesions have been reviewed elsewhere (56,57). Such fusion genes were thought not to occur in sporadic carcinomas until Chinnaiyan reported the presence of the TMPRSS2-ERG fusion gene in carcinoma of the prostate (58,59). Other similar fusion genes, e.g., TMPRSS2-ETVI, also have been identified in the prostate. Although controversial, some groups have reported that TMPRSS-ERG is an important prognostic factor for radical surgery of the prostate (60). Since the discovery of fusion protein in prostate carcinoma, several other fusion genes have been reported in lung and other carcinomas (61).

2.3. Actions of Suppressor Genes – Caretakers, Gatekeepers and Landscapers

Complex animals and humans successfully prevent the development of cancers via multiple mechanisms at the cellular, tissue (micro-environmental) and organismal levels. It may take several decades to arrive, via clonal selection, at even a pre-invasive neoplastic lesion and even these relatively advanced neoplastic lesions may regress by mechanisms which we are largely unknown. An excellent example is the mutations of p53 that are present in sun exposed skin. Skin carrying these mutations may represent up to 4% of the skin of a human (10,62) yet the pre-invasive neoplastic lesions which result from mutations in p53, actinic keratoses (AK), only develops in a small percentage of skin and even most AK lesions do not develop into invasive cancers; also, both clones with p53 mutations as well as AK lesions may regress (62–64).

The genetics of early neoplasia are quite complicated with various types of suppressor genes playing different roles, which are important at different times in the development of cancers.

2.3.1. Caretaker Genes—The caretaker genes are critical to the stable growth of tissues by producing products that maintain the stability of the genome. The same caretaker genes typically control the genome of multiple types of tissues and hence caretaker genes are usually not tissue specific (65–70).

Mutations or other changes in caretaker genes may lead to neoplastic changes; however, such changes in caretaker genes are, by themselves, insufficient to initiate the development of a tumor. Neoplasia arises only in a small fraction of cells having full defects in DNA-repair genes.

BRCA1 and BRCA2 are classic caretaker genes with mutations in both associated with an increased predisposition to breast and ovarian cancers; BRCA2 mutations also are associated with an increased predisposition to pancreatic cancer (71). Both BRCA1 and BRCA2 play a role in monitoring and/or repairing damage to DNA, most likely through the pathway that repairs double strand breaks in DNA and homologous recombination (72). Mutations in either of these two genes results in unrepaired damage in DNA. In the case of BRCA1, this may be due to the interaction with Rad51, which plays roles in DNA repair and recombination processes (72,73). Of interest, phosphorylated BRCA1 and BRCA2 and Rad 51 colocalize during the DNA damage response. Similarly, dysregulation or mutations in mismatch repair genes can result in hereditary non-polyposis colorectal cancers (HNPCC) as discussed subsequently.

The ATM gene, associated with ataxia telangiectasia, can be considered as a caretaker gene (74). The ATM encodes a protein that belongs to a family of protein kinases, and share similarities with the catalytic domain of phosphatidylinositol 3-kinases at its C-terminal region. As shown *in vitro* and in animal models, ATM is a key regulator of multiple signaling cascades that respond to DNA strand breaks. Cellular responses to the DNA damage involve the activation of cell cycle checkpoints, DNA repair and apoptosis. Exogenous carcinogenic agents, or normal processes, such as meiotic or V(D)J

recombination may induce the DNA damage. The ATM gene suppresses tumorigenesis in specific T cell lineages presumably through its caretaker function (74).

2.3.2. Gatekeeper Genes—In contrast to caretaker genes, gatekeeper genes are those genes that control the growth of tissues and cellular components within tissues. Thus, in their normal control of growth, they may inhibit proliferation, facilitate apoptosis and other forms of cellular death and/or promote terminal differentiation of cells (65–69).

Proliferation is controlled by the cell cycle which is regulated in part by specific signal pathways such as p16^{Ink4a}/pRb and associated cell cycle checkpoints (Cdk4-6 and cyclins D), the p14^{arf}/mdm²/p53 regulatory pathway of G1-S, the APC-β-catenin/TCF4, and the RUNX3 pathway (75). Also, there are interactions among these pathways, e.g., between pRb and p53 (69,70).

It is conceivable that gatekeeper genes may be rather tissue-specific and that the concept of gatekeeper genes applies to certain but not all tissue types. For instance, the RB, APC, RUNX3, NF1, MEN1, PCT, interleukin 12, SMAD4 and VHL genes are likely to be gatekeeper genes in their respective cell types (7,8,73–78), but ras genes, BRCA1 and BRCA2 are not (65). It is likely that gatekeeper genes could serve as useful tumor biomarkers because they are somewhat more tissue specific. For example, p16^{Ink4a} is involved in melanomas, pancreatic cancer, and squamous cell oral cancer. APC and RUNX3 are involved in colorectal cancer (75); SMAD4 is also a gatekeeper in oral cancer (7,8).

2.3.3. Landscaper Genes—Landscaper genes are genes that facilitate the growth of neoplastic lesions by creating a microenvironment that aids in unregulated cellular proliferation (77–84). Based on studies of combining malignant embryonic carcinoma cells from a teratoma with a blastocyst, it is clear that this micro-environment in which tumor cells reside exert the ability to “re-program” malignant cells so that they are controlled and act as normal cells (83). Similarly, stroma may induce neoplastic changes (78) in benign cells.

The interaction between stroma and epithelial cells is mediated by multiple components. Of these, the extracellular matrix (ECM) plays a very important mediating role (81). Signals may be transmitted between different cells via their direct interactions with the ECM, as well as via paracrine signals which can be modulated by the ECM which contains proteolytic molecules which can modulate and/or destroy signal molecules. Thus, the ECM acts to control unread signals of a neoplastic phenotype (77–81). Additional functions that landscaping genes may perform is a general inhibitory modulation of multiple types of signals needed for growth (77–82). The subsequent discussion of the microenvironment’s interaction with neoplastic processes is in the section of “microenvironmental influences in the development of neoplasia”. Loss of components of the ECM may lead to a microenvironment which can stimulate unregulated growth, clonal proliferation, and ultimately neoplastic lesions (77–83).

2.3.4. Deficits in Mismatch Repair or Microsatellite Instability—An important caretaker gene system is the mismatch repair system that functions to remove mismatched nucleotides and repair the sites of replication errors. A complex of MSH molecules recognizes the mismatched nucleotides and MLH1/PMS2 and MLH1/MLH3 complexes are involved in attachment, removal of the mismatch and repair. Overall, MSH2, MLH1, MLH3, PMS1, PMS2, MSH3 and MSH6 are involved in detection-excision and repair of mismatched nucleotides.

When there are mutations in MSH2, MSH6, MLH1 or PMS2 or loss of expression of MSH2 or MLH1 caused by, for example, methylation of the promoters of these genes, tumors may develop in the brain, colorectum, endometrium or ovary. The loss or dysregulation of mismatch repair in a patient frequently is referred to as a mutator phenotype (84).

Associated with these tumors are microsatellite changes at many positions throughout the genome. Microsatellites are composed of multiple adjacent copies of mono-, di-, tri- or tetra-nucleotides within the DNA. In microsatellite unstable cancers, the runs of nucleotides may be of different lengths than are the same microsatellite runs in germline DNA. Cases with mismatch repair are designated as having microsatellite instability or replication error rate phenotypes (84–88). The alterations in the length of simple repetitive sequences of nucleotides have been found associated with a distinct mechanism underlying carcinogenesis (85). Specific microsatellite markers are found in a broad variety of tissues undergoing long standing continuous damage, inflammation and repair, (LOCDIR), in pre-invasive neoplastic lesions and in tumors, while other microsatellite changes appear to be tumors specific (86). It is hypothesized that MSI is caused by a failure of the DNA mismatch repair (MMR) system to repair errors that occur during the replication of DNA. Failure of MMR is characterized by the accelerated accumulation of single nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences that occur ubiquitously throughout the genome (87–88). This hypothesis is supported by the study of HNPCC (hereditary nonpolyposis colorectal cancer) tumors and other non-hereditary colorectal tumors harboring MSI.

The loss of specific mismatch repair enzymes and the subsequent failure of MMR cause changes in multiple critical genes including TGF β R2, Bax, caspase 5, β catenin, PTEN, APC and β microglobulin (86). The changes in these and similar molecules lead primarily to hereditary non-polyposis colon cancer (HNPCC), but other tumors also occur in this setting. Of interest, HNPCC is not associated with large numbers of polyps, occurs primarily in the proximal (right) colon, and tends to be a less aggressive lesion than sporadic colorectal tumors (14,89).

2.3.5. The Effects of Epigenetics on Neoplasia—Epigenetics refers to changes in the expression of genes that can be inheritable, but the changes in gene expression are not related to the base sequence of DNA. For example, biochemical changes determine the part of the genome which will be transcribed. This is partially controlled by the nucleosome and the interaction of the nucleosome with chromatin structures during transcription. Specifically, histones of DNA are modified (e.g., acetylation) to determine activity of transcription and the packing of DNA (90–92). Histone acetylation affects the heterochromatin of the DNA within a cell and hence the ability to transcribe these areas of the genome.

Another major point of epigenetic control is the ability of cytosines that are 5' to guanosines, i.e., in CpG group of nucleotides to be methylated by a DNA methyltransferase enzyme. The methylation of CpG islands also causes various degrees of transcriptional suppression. Of interest, about half of the genes in the genome have promoters with CpG collections called CpG islands. Methylation of various components of the CpG islands together with histone patterns can act in gene silencing via methylation of CpG groups in their promoters and via the formation of heterochromatin (90–92).

Thus, the methylation of cytosine in CpG islands of DNA is an epigenetic process that may alter the function of genes without changing the base sequence of the DNA. The ability of methylation to block transcriptional activation is well documented, particularly within CpG-rich promoters (93). These types of promoters are known to be methylated at multiple sites, which may result in the inhibition of transcription or gene silencing. In cancer cells some

genes such as VHL and p16, have been found methylated and silenced, but only when exhibiting a wild type sequence (94). Also, silencing of the MLH1 mismatch repair gene by DNA methylation has been detected in colorectal tumors (95). Since it is unlikely that the mutational status directly could affect the occurrence of methylation, it was speculated that a low level of random methylation could produce the gradual methylation of CpG promoters whenever there is a wild type sequence exposed to constant selection for its progressive transcriptional silencing. This could also occur at sites of tumor-suppressors.

Hypermethylation of promoters may be frequent in some types of tumors, but is usually infrequent in normal tissues. Belinsky and colleagues (96) observed aberrant methylation of p16 as an early event in lung cancer and they proposed it as a potential biomarker for early diagnosis of lung cancer. Ongoing studies may elucidate whether aberrant methylation of p16 is common for other types of neoplasia. The Cairns laboratory found that 93% of 100 kidney tumors demonstrated promoter methylation in at least 1 of 10 genes evaluated. All grades and stages of kidney tumors were affected, but none of 15 normal kidneys or ureteral tissues demonstrated promoter methylation of any of these 10 genes (97). The pattern of promoter methylation seems to vary with tissue type, so this pattern might be tissue specific enough to be a useful tumor marker (98,99). Current studies also suggest that the patterns of promoter methylation might be useful in predicting responses to chemotherapy or chemoprevention (100,101). The discovery of numerous hypermethylated promoters of tumor-suppressor genes, along with a better understanding of gene-silencing mechanisms, underscore the importance of epigenetic mechanisms in tumor development and discovery of new biomarkers (100–105).

2.3.6. DNA/Protein Correlations—DNA may be prevented from directly being converted to equivalent protein levels via multiple pathways/mechanisms. In some cases, the mRNA may be very stable and only a few copies of mRNA are transcribed from the DNA. Usually in such cases the amount of protein available to cells is controlled by metabolism of the protein. An example of this is p27^{kip-1} which is primarily regulated post-transcriptionally by Skp-2; thus, these two proteins are usually inversely present in tissue (106,107). Alternatively, a mRNA may be very unstable and may be rapidly degraded. We know little about any mRNA with half life of a few minutes. Also, for some proteins to be functional, requires post-translational modification by, for example, the addition of sugar moieties. In addition, genes may be transcribed alternatively via various forms of splicing. An interesting example of such splicing is CD44 which has many splice variants of which CD44v6 is important as a marker of stem cells in breast cancer (108). Similarly, splice variants of other genes such as BRCA1 may modulate the function of these genes (109).

2.3.7. Modulation of mRNA—Recently, it has been recognized that the translation of a large proportion of mRNA molecules can be inhibited by single, small, non-coding RNA molecules that are composed of 21–24 nucleotides and that are called microRNAs (110–111). MicroRNAs (miRNA) are found in both plants and animals. In animals, the miRNAs partially base pair with the target mRNA speeding up its degradation (112–113); however, miRNA may less frequently act to inhibit the degradation of the target mRNA. Patterns of miRNA activity have been identified that distinguish between types of cancer and to identify more or less aggressive cancers (114). For example, loss of miR-133a and gain of miR-224 has been associated with the progression of colorectal cancer and miR-145 was reported to be down-regulated in metastatic colorectal cancer (115). Similarly, in prostate cancer (PCa) the expression of miRNAs separated PCa from benign prostate hyperplasia (BPH). Also, the miRNA expression was correlated with androgen dependence of samples of prostate cancer (116).

Other methods of by which mRNA is regulated endogeneously also have been reported, including the binding of proteins to mRNA; however, this form of regulation of mRNA has not been as extensively studied as microRNAs (117–119), but may be just as important.

2.3.8. Microenvironmental Influences in the Development and Progression of Neoplasia (See review in 120)

Non-malignant components of the microenvironment of tumors are critically important as to how a tumor develops. This includes tumor stromal interactions mediated by cytokines, tumor-immune interactions mediated by cytokines and exosomes and vasculogenesis/angiogenesis which are mediated by the cytokines, chemokines and stromal interactions (120). Many of the genes involved in the effects of the microenvironment on neoplastic processes are designated as landscaper genes.

Tumor-stromal interactions depend upon the neoplastic cells of the tumor, cells associated with the tumor such as inflammatory cells, especially macrophages, the fibroblasts and the conversion of fibroblasts to a carcinoma-associated fibroblasts/myofibroblasts. Each of the components of a tumor communicates with the other components, especially via their molecular secretions including cytokines and chemokines.

2.3.8.1. Tumor associated macrophages: Tumor-associated macrophages (TAM) are very important to the progression and metastasis of tumors. They stimulate angiogenesis via their production of VEGF, interleukin 6 and interleukin 8 and they stimulate tumor growth/invasion via TNF α and matrix metalloproteinases. In general, TAM do not function in the phagocytosis of tumor cells or in the presentation of tumor antigens to T cells (121–127).

2.3.8.2 Exosomes: Exosomes, the 40–100 nm vesicles secreted by various cells including the cells of tumors, are part of a pathway by which tumors communicate with and modulate the immune system (128) and potentially other tissues. The exosomal vesicles contain both cellular waste molecules and signal molecules. When exosomes fuse with target cells their signal molecules drive target cells to specific actions (128–131). Signal molecules include TNF α , TGF β and microRNAs. The suppression of the immune system has been monitored via decreasing the il-2 dependent proliferation of NK and T cells, blocking of T cell activation, induction of T regulatory cells and blocking of the maturation of dendritic cells (132–134).

With the blocking of dendritic cell maturation by exosomes and with the loss by TAMs of antigen presentation, the ability of the immune system to respond to tumor antigens would be greatly reduced.

The fibroblasts associated with the stroma of tumors are activated and become myofibroblasts; these produce vimentin and smooth muscle actin. Thus, these fibroblasts are referred to as tumor associated myofibroblasts (TAMF). TAMFs are similar to myofibroblasts associated with wound healing. Cells mimicking TAMFs may also be produced by epithelial-to-mesenchymal transitions (EMT).

TAMFs contribute to the malignant phenotype in multiple ways. This includes direct contribution by perhaps conversion of adjacent uninvolved epithelium to cancer (135,136) and by indirect effects via the secretions of landscaper genes such as S100A4, il-6 and il-8 which may potentiate cellular motility, can induce angiogenesis, and may modify the stromal environment (137–139). TAMFs and TAMs also secrete CXCL12, TGF β , and MMP-13 (140–142). CXCL12 stimulates the proliferation of some types of tumors (e.g., prostatic carcinoma {141}) and potentiates their metastatic spread (e.g., prostate carcinoma {142,143}). The CXCL12–CXCR4 interaction has varying effects depending on the type of prostate cell. In general, this interaction results in increased stromelysins 1, 2 and 3 as well

as MMP (142). CXCL12 also is a chemoattractant for leukocytes and hence contribute to inflammation which affects the progression of some types of cancer.

2.3.8.3. Angiogenesis: Angiogenesis is one of the 6 original hallmarks of cancer (18) in that it is required to provide nutrients and oxygen to cancer cells which permit growth and survival of cancer. There are multiple factors which modulate angiogenesis – both stimulatory and inhibitory factors of which VEGF (VEGFA) is one of the most studied. The stimulatory factor VEGF and similar splice variants act by binding to the VEGFA receptor (144–146) to increase the migration and rate of proliferation of vascular endothelial cells; it also creates openings in blood vessels to increase vascular permeability and causes vascular lumens to develop. Most cancers including breast cancers express VEGF and its receptors, KDR or FLT1, and the extent of its expression correlates positively with a poor outcome and more rapid recurrence. Also, VEGF may act to block the beneficial effects of drugs on cancers (147,148).

VEGF like many angiogenic factors is stimulated by hypoxia via a complex of HIF1 α with HIF1 β . IL-6 and IL-8 also are stimulated by hypoxia as well as levels of glutamine (139). IL-8 is released via stimulation by NF κ B and Activating Protein 1, it then acts as an angiogenic agent similar to VEGF (149–153). Other angiogenic agents include TGF α , bFGF, angiogenic transcription factor 1 (ETV-1), interferon inducing protein 10 (IP-10), and CXCL1 (144,146,154,155).

Of interest, clones may develop without chromosomal or genetic instability. This has been demonstrated by studies in which clones of cells are grown via multiple passages after they have exhibited growth that is non-contact inhibited or the same clones for comparison are grown in low density passage. After 20 or so generations of passage of non-contact inhibited cells, but not of low density passage cells, the cells can produce malignant tumors when transplanted into a syngenic mouse. This transformation of a clone to a frankly malignant tumor is because the clone has taken advantage of its growth advantage as well as has adapted to its micro-environment. This clonal growth advantage may be via autocrine stimulations in which the group of cells now have established one of the hallmarks of cancer – the development of a continuing self-stimulatory environment (18). Upon transplantation into a syngenic animal, this self stimulatory environment continues and in addition, the clone may develop paracrine or exosomal pathways of stimulation or may be stimulated by the components of the surrounding micro-environment.

2.3.9. Clonal Selection—A clone of cells, by definition, is a group of cells growing together that have arisen from a single cell, usually a stem cell, that has developed secondary to a growth advantage to both the parent and offspring cells. Some of the characteristics of the cell of origin are uniformly carried by the offspring cells which permits the identification of the group of cells as a clone. Clones of cells are most easily detected in tissue when the members of a clone carry a marker related to the transformation such as a mutated p53 (10,17) in culture or when they have a marker which is a physiologic characteristic such as increased proliferation that is not contact inhibited. Clones in tissue tend to be relatively small, usually with less than 1000 cells unless they form a pre-invasive neoplastic lesion. Most studies of clonal characteristics have been in culture, where culturing clones that have escaped contact inhibition has led to increased knowledge of the behavior of individual clones.

Clonal development is thought to be one of the main factors in the development of neoplastic lesions. First, there is either a change in the cells of the clone or a change in the microenvironment. In response to either type of change, there is a growth advantage to the members of the clone. As the clone expands, genetic and epigenetic changes add

heterogeneity to the clonal members. This is typically followed by additional selection and additional evaluation until the hallmarks of cancer develop (18).

Most, if not all cancers are monoclonal, but not all actively growing clones of cells are recognized as pre-invasive neoplastic lesions. Genetic markers that can detect the clonal outgrowth of neoplastic cells may be used for the detection of primary pre-invasive neoplastic lesions and evaluation of early tumor development. Since monoclonality is often a marker of evolving neoplasia, it could be used to detect new neoplastic lesions or monitor evolution of the pre-existing tumors. As clones continue to expand, genomic instability may develop; this might be followed by the development of pre-invasive neoplasia depending upon clonal characteristics (156–162).

2.3.10. Viral Insertion—Integration of a viral genome into a human host cell can lead to a variety of interactions with their host cells that may be relevant for the induction of cell transformation, the maintenance of a transformed phenotype, and tumor progression (163). Identification of novel viruses associated with early stages of neoplastic transformation could lead to new biomarkers and more effective preventive strategies. Several DNA and RNA viruses have been implicated as causative agents or cofactors in certain forms of human cancer. For example, the association of human papilloma viruses (HPV), hepatitis B virus (HBV), and Epstein-Barr virus (EBV) with human cervical, hepatocellular, or nasopharyngeal carcinomas, respectively, has been extensively documented (see The Biology of Incipient, Pre-invasive or Intraepithelial Neoplasia, Grizzle WE, Srivastava S and Manne U in Chapter 1 of this text). Unlike retroviruses, which are obligate mutagens because their replication cycle and persistence require integration into the host chromosomal DNA, the integration of certain DNA viruses into the chromosomal DNA was once not considered a requirement for viral persistence. This view is challenged, however, by evidence for HPV's integration in most invasive genital cancers, HBV integration in the majority of hepatocellular carcinomas and EBV persistence in integrated form within infected lymphoblastoid cells or Burkitt's lymphomas.

3. APPLICATION OF BIOMARKERS TO UNDERSTANDING NEOPLASIA

Biomarkers may reflect distinct stages of the neoplastic process and could be used in a variety of applications. Potential clinical applications are implicit in the term “biomarkers”, which is defined as morphological, biochemical, or genetic alterations by which a physiological or pathological process can be identified or monitored. Potential uses of cancer biomarkers include the following: early detection of neoplastic lesions, distinguishing pre-neoplastic from pre-invasive neoplastic lesions and monitoring patients with established cancer for recurrence, development of metastases or a second primary tumor (164,165). Further, biomarkers can be used for the assessment of risk for developing cancer and establishing surrogate endpoints for primary or secondary prevention trials (166). Biomarkers also can be used to predict responses to novel or other therapies. To be clinically useful biomarkers must have high predictive accuracy, and must be easily measurable and reproducible. Tests for biomarkers in most settings must be minimally invasive, and acceptable to patients and physicians. Each of these uses have been described in a chapter of this text, “Translational Pathology of Neoplasia”, Grizzle WE, Srivastava S and Manne U.

4. FUTURE DIRECTIONS IN THE ANALYSIS OF BIOMARKERS

4.1. Development of highly sensitive and specific biomarkers

It is likely that early detection strategies based on biomarkers could have a great impact on cancer rates. The challenge is to develop new scientific and research approaches in early detection in order to develop more sensitive and accurate intervention strategies with the

highest impact on incidence and mortality. In addition, such a biomarker should be detectable early in the carcinogenic process and should be associated with the risk of developing cancer or the occurrence of precancer. Ideally, the biomarker should be detectable in body fluids and/or in tissue obtained via minimally invasive methods such as fine needle aspiration (167). Biomarkers of early detection should be potentially modifiable by preventive agents. Any biomarker that is useful clinically should be suitable for development of adequate quality control procedures.

Any marker to be used for population-based studies must be accurately characterized with respect to sensitivity, specificity, positive and negative predictive value, accuracy and reproducibility. Planning for evaluation of molecular markers in clinical trials must also consider the morbidity and costs that may arise from the follow up of individuals who test positive (160,168). The use of highly sensitive but less specific tests may result in a large number of false-positive individuals who require further diagnostic examination. For instance, it is estimated that PSA screening of all individuals age 50–70 would result subsequent diagnostic and therapeutic procedures costing up to \$27.9 billion in the first year, as reported in 1994 (168).

The phenomenon of field effect could be used to evaluate the risk of cancer. Many cancers, e.g., head and neck, develop in fields of primed epithelium. Identifying field changes through the application of biomarkers could represent a very early stage of detection; however, if a pre-invasive neoplastic lesion or an SCC develops in the oral cavity, the field effect is established. Also, either the whole oral cavity would require preventive therapy or a watchful waiting approach could be taken until pre-invasive neoplastic lesions develop which could then be treated individually. As new, sensitive technology evolves and our understanding of pre-invasive neoplasia improves, future research should focus on the development of new biomarkers to evaluate the risk in less accessible organs. For instance, predisposition to cancer in non-accessible target organs, such as the lung, could be evaluated by determining the genetic changes that occur in readily accessible organ sites, such as the buccal mucosa, with which the non-accessible target organs form an anatomic and/or a functional continuum because they have been exposed to similar carcinogens (165). Recently Roy et al., using light scattering technology, has been able to identify colorectal neoplasia based on field effect changes that are caused by cancers and modify the light scattering patterns of uninvolved epithelium (169–171). Importantly, this approach has been used to identify proximal neoplastic lesions by light scattering patterns in the distal rectum (172). Thus, this approach, though not understood, may represent a great advance in screening for colorectal cancer.

Of great value for early detection and risk assessment of various malignancies would be the identification of markers detectable in body fluids and various specimens other than tissue, for example sputum, saliva, urine, stool, blood and breast nipple aspirates. Identification of tumor markers --both DNA, RNA and protein based-- in body fluids suggests the possibility for developing markers for large-scale screening and risk assessment. To detect DNA-based markers, derived from tumor cells, highly sensitive methods are needed, because tumor cells are greatly outnumbered by normal cells in blood, stool, saliva, and other potential assay targets. It seems, however, that DNA-based markers are less applicable in blood-based screening tests for early lesions (173), because circulating tumor-derived DNA has been generally found associated primarily with advanced tumors. In contrast, proteins detectable in serum and other body fluids might be excellent markers for the detection of localized lesions, presumably early neoplastic lesions. Ideally, biomarkers should be easily measured in body fluids that may be accessible for multiple longitudinal sampling. There is a great need to improve early detection and to identify prognostic biomarkers; this includes

the identification of malignant transformation, progression or invasion, and prediction of responses to prevention including chemoprevention.

Proteomics-based approaches to detect biomarkers may have distinct advantages over DNA and RNA-based techniques in that they give direct evidence of abnormal gene expression at the time of sampling. In addition, some biomarkers as discussed are regulated post-transcriptionally and hence cannot be detected at the mRNA level; however, miRNA offers a new potential group of biomarkers that currently are being evaluated. Proteomics also offers development of tests for use in routine clinical practice. DNA, RNA and protein-based research is complementary. Identification of DNA sequences coding for specific proteins can stimulate subsequent expression analysis in tissues or biological fluids.

Approaches to the development of cancer related biomarkers suitable for clinical application have been fragmented and sporadic, resulting in data of limited practical value. Usually the results of studies of biomarkers published in the literature cannot be generalized to the population as a whole. They usually are not performed in defined populations, nor are they even prospective. Systematic studies designed to improve sensitivity, specificity and high throughput of cancer related biomarkers have been rather limited until recently. A considerable barrier which has prevented research in validation of biomarkers has been the limited availability of good quality tissue specimens from normal, early neoplastic and advanced neoplastic lesions, along with respective body fluids and demographic and clinical follow up data. The Early Detection Research Network (EDRN) has established multiple collections of specimens of bodily fluids via which potential biomarkers can be tested. These samples are available to both EDRN and non-EDRN investigators (see the EDRN website, <http://edm.nci.nih.gov/>).

4.2. Performance Characteristics

The systematic approach to the identification and development of biomarkers entails several important issues. First, a laboratory performing the study must be able to reliably measure biomarkers in specimens accessible to oncologists and epidemiologists. Biomarker assays that are performed should detect the specific abnormality in a high proportion of cancer patients (it must be sensitive), but in very low proportion of non-cancer control individuals (it must be specific). Finally, a reasonably large and representative cancer and control group should be available for evaluation to allow precise interpretation of test results and avoid bias (165,166,174–176). The selection of an appropriate control group may be difficult without introducing bias. Optimally, the same number of cases and controls should come from each site and the various conditions of sampling should be very similar. Avoiding bias has become much more important as the use of multiplex methods has increased. As the number of measurements in assays increase, the effects and likelihood of bias increased. Methods using mass spectrometry are especially prone to bias because individual multiple peaks of a complex spectrum may constitute a measurement (176,177).

The choice of biomarkers as biomarkers of early cancer will depend on their performance characteristics, which include accuracy, sensitivity, specificity and reproducibility. To be suitable for screening for early detection of neoplastic lesions (asymptomatic and high risk subjects), biomarkers must be able to detect disease when it is present, and to identify those patients without the disease (specificity) (Table 4). Ideally, biomarkers must have both a high sensitivity and high specificity. The sensitivity and specificity should be balanced to increase specificity and to avoid large numbers of false positives. This is a major consideration when biomarkers are used to screen a population with low disease prevalence in which a positive test can trigger invasive or very costly investigations. A reference value may be chosen to balance the sensitivity and specificity of a biomarker-based test that is based on the prevalence of the disease in the population. For example, the prevalence most

cancers is less than 1%. With the reported sensitivity of 99% and specificity as high as 99% for a single assay, half of all patients screened will not have the disease, but would be positive for the test. Only cancers with prevalences of greater than 1% will have less false positives than true positives.

Table 4:

Prevalence = 1% or 100/10,000 cases

Sensitivity – 99%; specificity 99%

Test	+	–	TOTAL
Cancer	99	1	100
Non-cancer	99	9801	9900
TOTAL	198	9802	10,000

Incidence is the number of patients diagnosed per year with the disease. **Prevalence** is the number of individuals with the disease existing in the population. Incidence and prevalence are not the same and incidence will always be less than prevalence; however, the true prevalence of a disease is usually unknown.

Prevalence example 1%	1000/100,000	Total in male or female population
Incidence		
Lung/bronchus	87.3/100,000/yr	Males only considered
Prostate	158.2/100,000/yr	Males only
Breast	123.6/100,000/yr	Females only

One approach to achieve a satisfactory cut-off or reference value for a screening test is to use a receiver operating characteristics curve (ROC); this is a plot of the true positive rate (TPR) versus the false positive rate (FPR). The higher and farther left the ROC curve, the better the performance of the test. The curve provides the ability to establish cut-off values in which only values greater than or equal to the cut-off values are called positive. For each pair of cut-off values, one can estimate FPR or 1-specificity and the sensitivity or TPR.

Both large retrospective studies and prospective studies have to be performed to validate promising markers for clinical usefulness. Retrospective studies that utilize archival pre-invasive neoplastic lesions, such as dysplasia or in situ carcinoma, may rapidly determine the presence and frequency of a given molecular alteration in early stages of the neoplastic process. This can be the first step toward ultimate validation of clinical utility. Markers identified in pre-invasive neoplastic lesions are good candidates for further characterization and for evaluation for their effectiveness in detecting cancer at an early stage. Prospective randomized studies may determine whether a promising marker, which recognizes pre-invasive neoplastic lesions, is specific and sufficiently sensitive to accurately predict the development of cancer and lead to improvement in cancer-specific mortality.

A biomarker of an intermediate endpoint can be validated within a chemoprevention trial. Optimally, both a valid and useful marker would be modulated in the short term, within one year, and that modulation would correlate significantly with reduction in cancer incidence in

long term, e.g., five to ten years. Such performance standards would validate a marker as an indicator of a clinical and histological outcome for a given type of tumor and a specific drug, but may not be generalizable to other populations at risk or other drug classes.

Eventually, a marker should be validated in various systems, each involving a different type of neoplastic lesion and different type of drug in order to assess the degree of its generalizability. It has been suggested that a panel of valid biomarkers which are associated with the intermediate points of carcinogenesis may be more useful for determining the efficacy of chemoprevention than any single marker alone (178,179). As the number of genetic markers increases, complex statistical issues arise which translate into practical clinical considerations. If a large number of markers are tested, the probability that any one is positive in a normal person increases causing frequent false-positive associations. Such problems must be considered and corrected statistically. Clear examples of markers that are more likely to be useful for a wide variety of uses (e.g., surrogate endpoints, prognosis, early detection) are markers of proliferation and apoptosis.

4.3. Clinical Validation

Although many biomarkers for early detection of neoplastic lesions and assessment of cancer risk appear promising, no marker has been fully validated for its ability to identify early neoplastic lesions, to predict cancer risk or to predict responses to chemopreventive agents (180,181). To do so, biomarker validation studies should be incorporated into prospective clinical trials. Marker validation in the chemoprevention setting should begin with controlled clinical studies, new or ongoing, consisting of a distinct type of neoplastic lesion and a specific treatment. The feasibility of biomarker evaluation should be based on several criteria, which include 1) differential expression of the biomarker in normal high risk tissues versus pre-invasive neoplastic tissues versus cancer; 2) ability to analyze the biomarker in small tissue specimens; 3) quantitative level of biomarker phenotypic expression correlating with distinct stages of carcinogenesis; and 4) availability of clinical and preclinical data supporting the modulation of biomarkers induced by the study agent. As suggested by Lippman (178), the evaluation of a surrogate endpoint within chemoprevention trials should consist of several stages. In the first stage a non randomized short-term trial in high risk population with or without pre-invasive neoplastic lesions should be performed to determine the feasibility of the study and to select or prequalify candidate markers.

The use of a high risk population in early prospective studies is recommended in order to have a sufficient number of expected events in a relatively small sample size of subjects. In the second step of a validation process, a nontoxic dose and schedule trial should investigate modulation of biomarkers of intermediate endpoints to further define supportive preclinical and epidemiological data and to improve the rationale for the third step of validation. Ultimately, the third step of validation should be a long-term phase III trial using cancer incidence, a true endpoint, as the endpoint. It is expected that this step of validation could demonstrate a strong association between modulations of the biomarker and the risk of developing pre-invasive neoplasia and ultimately, cancer (182).

Similarly, the steps in a validation trial for a marker(s) of early detection have been described by Pepe et al (183). These are divided into 5 phases which should be completed before a biomarker for early detection can be used clinically. These include 1) Preclinical Exploratory Studies; 2) Clinical Assay Development for Clinical Disease. For an assay to be considered to be promising, this phase should distinguish patients with cancer from those without cancer. This phase does not detect early cancer because the samples from patients with cancer are considered from patients with established cancer; 3) Retrospective Longitudinal Study – This study should use samples obtained prior to diagnosis of either cancer and no cancer (control) subjects. Control subjects are defined as having related

subject characteristics and to be cancer free during a specified time of follow-up; 4) Prospective Screening Studies – The goal of phase 4 is to determine the effectiveness of the screening test prospectively in a chosen population. Of importance, is determining the rate of detection and the false referral rate (false positive rate); 5) Cancer Control Studies – This final study is to demonstrate that use of the test reduces cancer mortality via the early detection of cancer. Successfully completing these steps are likely to ensure that this test will be used clinically. It is likely that the process of population-based screening and intervention will evolve through gradual refinements and improvements of technologies and better analytical tools for basic, clinical and epidemiologic information. This last step is in general beyond that necessary for approval by the FDA.

There have been several larger studies which have included or will include components to determine the effectiveness of biomarkers in detecting cancer such as the Prostate Cancer Prevention Trial and Prostate, Lung, Colorectum and Ovarian Cancer Screening Trial. These have had to overcome difficulties with obtaining appropriate samples from cases and controls. Several approaches and technical developments should aid in future studies. There are now several resources to aid in studies of biomarkers in early detection: First, the PLCO is providing longitudinal samples of serum on patients who have developed a specific cancer. These samples can be very useful in studies focused on Early Detection (<http://prevention.cancer.gov/programs-resources/groups/ed/programs/PLCO/about#top>). Second, the EDRN has established a collection of samples of serum and sets of aliquots of plasma from cases and controls related to various cancers and urine for the study of bladder cancer. These samples are available and provided as unknown de-identified sets of samples for Phase 2 studies of biomarkers. After an investigator completes the analysis of a set of samples, the investigator returns the results to the EDRN which evaluates the results as to whether or not the biomarker is promising as a clinical marker (<http://edrn.nci.nih.gov/about-edrn/>).

4.4. Technology for Biomarkers Development

Even the most promising new markers have been still limited by technical difficulties and the probable high cost of implementing their use. The ability to lower cost and improve efficiency can greatly accelerate testing of new generations of biomarkers in screening settings in which lower costs and automation are highly desirable.

Several new technical breakthroughs not only will aid in the conservation of existing samples from cases of cancer and controls, but also expand the utilization of new types of samples. These include multiplex immunoassays, tissue microarrays, TaqMan Low Density Arrays (TLDA) for real-time quantitative – polymerase chain reactions (RT-Q-PCR), hybridization chips for expression of single nucleotide polymorphism (SNP) and DNA sequencing. Current multiplex immunoassays permit the analysis of many antigens concomitantly on a relatively small sample (100 µl) of serum, plasma or urine. Similarly, small samples of tissue, e.g., fine needle aspirates (FNA) may be homogenized and multiple antigens or genes can be analyzed concomitantly on the same sample.

TLDA analysis also permits multiplex analysis of large numbers of genes on multiple samples. For typical TLDA analyses one can analyze 48 genes including housekeeping genes on 8 samples, 96 genes on 4 samples, 192 genes on 2 samples or 384 genes on 1 sample. Also, it has been demonstrated that by using small primers of less than 100 base pairs, degraded RNA can be measured. This permits RNA to be measured using RT-Q-PCR in formalin fixed paraffin blocks; the results are equivalent to measuring the RNA in matching frozen tissue (184,185). For example, this is the approach by which the aggressiveness of node negative breast cancer subtypes are measured by the *Oncotype DX*®

test (186–188). The development of *Oncotype DX*® test is a good example of how various tests are now finding their way into clinical types of use.

Single nucleotide polymorphisms are genetic variation in single bases of DNA of an individual or a population. In general, there is one SNP per 100 to 300 bases; in general SNPs are excellent geographic markers for DNA in that they are located both in non-coding regions and in regulatory regions. SNPs that cause non-conservative changes in coding may affect the function of genes.

New technologies have rapidly developed for measuring SNPs. Some service sites can now analyze 10,000 SNP genotypes per day; throughput is increasing yearly and costs are decreasing. Not only can SNPs be determined, but also the copy number can be measured concomitantly (189).

Automation now permits cost effective and rapid identification of gene mutations, deletions, amplifications, or expression patterns in pre-invasive neoplastic lesions and cancer by direct sequencing. Through simultaneous analysis of the expression pattern of thousand of genes, it is possible to investigate whether the majority of differentially expressed genes are tumor specific or cell type specific, and whether most differences are intrinsic to tumor cells or dependent on the tumor microenvironment. Similarly, this methodology could greatly facilitate the detection of early lesion-specific probes which could, in turn, lead to the development of DNA, RNA, or protein-based assays for large scale, population-based screening.

5. SUMMARY

Studies using such biomarker methods as PAP screening, mammography or fecal occult blood testing have shown that detection of early cancer can reduce morbidity and mortality. Nevertheless, these established technologies as well as the PSA test are limited by suboptimal sensitivity and specificity, as well as high costs of screening and/or of dealing with false positive results. Therefore, it seems reasonable to explore the application of the new molecular-based technologies for earlier and more specific detection of neoplastic lesions and even for assessment of risk, that is, identifying patients who are likely to develop neoplastic lesions before cancer physically develops. The determination of risk is necessary in order to institute prevention.

The application of molecular methods in the field of early cancer detection is a dynamic process continually driven by expanding knowledge of carcinogenesis and by the availability of more functional and higher throughput technologies. At the molecular level, the detection of earlier cancer will require a more complete understanding of the evolution and regression of pre-invasive neoplastic lesions and associated molecular and genetic changes.

As we have studied the use of biomarkers in translational studies of cancer, we have realized that no single biomarker is likely to be useful as a specific biomarker in detecting early cancers, in risk assessment or in determining prognosis. However, the phenotypic expression of single molecular species are still very useful in pathologic diagnosis of tissue (e.g., prostatic specific antigen). An important development and example in this area is the development of the *Oncotype DX*® approach to analysis of the aggressiveness of subtypes of breast cancer. Similarly, as the cost of complete sequencing decreases, the speed-throughput increases, and our knowledge of genetic function increases, the ability to analyze a whole genome to evaluate, for example, the risk of developing a specific cancer or the determination of the aggressiveness of specific tumors become more feasible and likely.

The challenges of earlier cancer detection are multi-disciplinary in nature including issues in basic science, the development of technology, the finding of key genetic changes, and the statistical analysis of changes in multiple gene functions. Increasing integration of medical knowledge and new advancements in various areas of research, including medical oncology, pathology, molecular and cellular biology, molecular epidemiology, genetics, informatics, physics, statistics, and bioethics provide unprecedented opportunities for discovery and development of biomarkers and for understanding of neoplastic process. New scientific approaches which utilize genomic technology and bioinformatics may afford critically important insights into both hereditary and sporadic forms of neoplasia, and revolutionize methods for early detection of pre-invasive neoplastic lesions, risk assessment and prevention. Integration of technology, science and informatics is a key element for accelerating development and application of biomarkers. A critical aspect for better early diagnosis of cancer is the improvement of detection and characterization of early neoplastic lesions. A primary strategy is to facilitate an ongoing interaction between basic scientists, oncologists, clinicians, pathologists, geneticists, theoretical and applied biostatisticians, epidemiologists and other health professionals, because this is critical for the successful application of new discoveries in molecular biology for earlier detection of neoplastic processes. In addition, biomarkers are likely to play key roles as translational research aids the transition of medical care to personalized medicine.

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Fig. 1. The development of micro-invasive pancreatic cancer via the development and progression of pancreatic intraepithelial neoplasia. (PanIN).

Fig. 2.
Early Detection Tunnel: early detection of neoplastic lesions impacts on efficacy of intervention and survival.

Table 1

Precancerous Lesions and Conditions

Lesion type	Examples
Non-Neoplastic Precancerous Lesions	Endometrial hyperplasia Barrett's esophagus Intestinal metaplasia Atypical hyperplastic lesion in breast Squamous metaplasia (Bronchus) Cirrhosis of liver
Precancerous Conditions	Inflammatory bowel disease (e.g., ulcerative colitis) Chronic gastritis Chronic pancreatitis Chronic cholecystitis Leukoplakia of oral cavity
Pre-invasive Neoplastic Lesions (Dysplasia, Intra-epithelial Neoplasia)	Prostatic intraepithelial neoplasia (PIN) Esophagus (squamous or columnar epithelial dysplasia) Gastric (adenomas) Colonic (adenomas and flat adenomas) Endometrial intraepithelial neoplasia (EIN) Cervical intraepithelial neoplasia (CIN) Ductal or lobular carcinoma in situ (DCIS/LCIS) Pancreatic intraepithelial neoplasia (PanIN) Oral cavity (squamous dysplasia) Larynx (squamous dysplasia) Lung (bronchoalveolar in situ carcinoma, atypical adenomatous hyperplasia, squamous dysplasia)

Table 2

Molecular Alterations Commonly Found in Human Cancer		
Type of Mutation	Possible Causes of Mutation	Possible Consequences of Mutation
Single Base Change	Carcinogens Defective repair genes	Missense: may alter gene function Nonsense: truncates gene
Methylation	Defective methylation gene	Gene silencing or inappropriate expression
Small insertions or deletions	Carcinogens Defective DNA repair genes	Frameshift: truncates gene
Microsatellite instability	Defective DNA repair genes	Frameshift when inside gene Alters mRNA stability; differentially effects specific genes, e.g., TGFβRII
Large deletions	Defective DNA repair genes Defective DNA replication genes Illegitimate recombination Double strand break, with incorrect rejoining Homologous recombination	Loss of gene function of multiple genes
DNA amplification	Defective DNA repair genes Defective DNA replication genes Illegitimate recombination	Overexpression of gene

Table 3

Biomarkers involved in regulation of proliferation, differentiation, apoptosis, senescence and DNA repair detected in premalignant neoplastic lesions

Examples of Biomarkers in Premalignant Lesions

s

Proliferation

Ki67

PCNA (Proliferating Cell Nuclear Antigen)

Creatine kinase

Cyclin E

Cyclin D

Bcl-1

Mitotic rate

Oncogenes

p185^{erbB2}

cKi-ras

c-myc

erbB1(mutated EGFr)

H- or K-ras mutations

Tumor suppressor genes

p53

Rb

p16 (Ink4)

p19 (Arf)

Growth factors

TGF- α

EGFR

TGF β

TGF β R1

TGF β R2

IGF1R

IGF2R

ERP28

Differentiation

CD44v6

CDw75ag

Apoptosis

Bcl-2

Bax

Senescence

Telomerase

DNA Repair

MSH3

Examples of Biomarkers in Premalignant Lesions

s

MSH6

PMS1

Table 4

Clonality as Biomarkers of Neoplasia			
Molecular Alteration	Organ Site	Type of Neoplasia (detection)	References
Allelic loss	Head and Neck	Second primary	[91]
Microsatellite instability	Head and Neck	Unknown primary	[92]
Allelic loss	Head and Neck	Recurrence of cancer	[93]
Microsatellite instability	Head and Neck	Second primary	[94]
Microsatellite instability	Lung	Early cancer	[95]
Loss of loci	Lung	Second primary	[96]
Mutation	Lung	Early cancer	[97]
Microsatellite instability	Bladder	Primary tumor	[98, 99]
Microsatellite instability	Bladder	Recurrence of cancer	[100]
Microsatellite instability	Colorectal	Early cancer	[101]
Mutation	Colorectal	Early cancer	[102]
Microsatellite instability	Colorectal	Early cancer	[103]
Mutation	Gallbladder	Primary tumor	[104]
Microsatellite instability	Gastric	Early cancer	[105]
Mutation	Ovarian	Early cancer	[106]