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Incorporating Pathologists' Criteria of Malignancy Into the Evolutionary Model for Cancer Development

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Abstract A wide variety of alterations in cell and tissue structure still form the basis for cancer diagnosis by pathologists. Cancer development is recognized to be an evolutionary process [Foulds, 1954; Cairns, 1975; Nowell, 1976; Sager, 1982; Tomlinson et al., 1996; Cahill et al., 1999; Tomlinson and Bodmer, 1999], but the phenotypic changes diagnostic of cancer (pathologists' ''criteria of malignancy'') have not been integrated into the existing evolutionary framework. Since phenotypic changes bear an important relationship to the genetic and physiologic changes underlying Darwinian evolution, we propose that diagnostic structural alterations also bear an important and predictable relation to both the cancer genes and the functional alterations active at any particular step in the development of a cancer. Cancer genes are predicted to mediate the acquisition of cellular-level diagnostic criteria and the diagnostic cellular-level structural changes should reflect in a useful manner the altered cell physiology required for the cell to achieve increased ''cellular fitness'' at any particular step of colonal evolution. Tissue-level criteria of malignancy should relate less directly to specific cancer genes, but tissue-level criteria should still provide essential insight into the interplay of the altered cellular fitness with the constraints imposed by the cells' microenvironment. The evolutionary framework allows tissue-level criteria of malignancy to be expressed in terms of viable hypotheses for the mechanism of clonal expansion at any particular step in cancer development. This approach to conveying the tissue-level criteria of malignancy complements pattern recognition approaches to diagnosis, and establishes common ground between pathology and cell biology. When viewed from this perspective, the functions of cancer genes appear quite different from those predicted by the ''Gatekeeper, Caretaker'' or ''Hallmarks of Cancer'' models. Finally, a full evolutionary framework incorporating the criteria of malignancy restores congruity between the histogenetic classification and the emerging molecular classification of cancer. J. Cell. Biochem. 93: 28–36, 2004.

Key words: carcinogenesis; nuclear structure; tissue; histopathology

"On the belief that this is a law of nature, we can, I think, understand several large classes of facts . . ." (Darwin [1859]: The origin of species (1998): 129p]).

CANCER AS AN EVOLUTIONARY PROCESS

Evolution is the consequence of natural selection acting on heritable variation in fitness. For the clonal evolution of cancer, heritable variation can be due to mutations and epigenetic changes such as altered DNA methylation or histone modification [Plass, 2002]. Hybrid formation between tumor cells and normal cells could theoretically generate diversity in heritable fitness and promote clonal evolution [Pawelek, 2000] similar to the way sexual reproduction facilitates Darwinian evolution [Futuyma, 1998]. Otherwise, clonal evolution of cancer is asexual and akin to "adaptive" or "sympatric" speciation, allowing the complexities of speciation by reproductive isolation as well as the whole field of sexual selection to be conveniently ignored [Futuyma, 1998].

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Cancer genes (used in the broadest sense to include oncogenes and tumor suppressor genes) mediate the evolution of cancer cells, so cancer genes can be envisaged to function by increasing "cellular fitness." The term "cellular fitness" is useful because it appeals to a common-sense understanding of Darwinian evolution, and it correctly implies a broad range of mechanisms. Increased "cellular fitness" means that a heritable event has allowed clonal expansion.

GENETIC INSTABILITY AND CANCER

It is currently debated whether or not genetic instability is required for the acquisition of multiple heritable changes within a monoclonal tumor [Loeb, 1991; Tomlinson and Bodmer, 1999; Tomlinson et al., 2002]. Certainly genetic instability exists in many common cancers, and several useful cytologic criteria of malignancy reflect aneuploidy and genetic instability, for example cell-to-cell variation in the degree of chromasia, abnormal mitotic figures, and gross nuclear asymmetry [Fu et al., 1981]. However, several forms of cancer (including papillary thyroid carcinoma [Roque et al., 1995; Nikiforov et al., 1998], many sarcomas [Sreekantaiah et al., 1994], and hematopoietic malignancies [Le Beau and Rowley, 1986]) appear to show few genetic or epigenetic [Huang et al., 2003] abnormalities, as if genetic instability were not required. Whether or not genetic instability ultimately is required for development of any particular cancer, mutation by itself is insufficient to describe evolution [Cahill et al., 1999]: natural selection must still act on heritable phenotypic variation for adaptive evolution to occur. Likewise, mutations in "caretaker" genes (that promote genetic instability [Kinzler and Vogelstein, 1997]) do not explain how clonal evolution occurs. In the arguments that follow, we are concerned only with the mechanisms for increased cellular fitness and clonal expansion, rather than mutations or genetic instability.

A "GATEKEEPER" MODEL FOR CANCER GENE FUNCTION IS INSUFFICIENT TO DESCRIBE THE MECHANISMS OF CLONAL EVOLUTION

Interest in a cell-kinetic or "gatekeeper" [Kinzler and Vogelstein, 1997] explanation for cancer derives from the mathematical fact that one or more of the following three conditions must be met for clonal expansion: cancer cells must replicate faster, a smaller proportion must die, or a greater proportion of daughter cells must replicate compared to the normal cells from which they arose [Mendelsohn, 1960; Baserga, 1965; Tannock, 1978]. In fact, the third condition provides by far the most common, if not exclusive, explanation for accrual of cancer cells. The cell cycle time of even the fastest-growing human tumor, for example an acute leukemia, is longer than the normal stem cell counterpart [Baserga, 1965; Tannock, 1978], and cell death is frequent in tumors. Pathologists are aware of the problems of a cell accounting model: mitosis is not a useful criterion for diagnosing many forms of cancer. For example, mitotic figures are essentially irrelevant for establishing a diagnosis of breast cancer, or malignancy in an effusion [Rosai, 1996]. Paradoxically, cell death is sometimes a criterion of malignancy (for example, in breast cancer and sarcomas [Rosai, 1996]).

It is not a paradox that cancer cells do not have to either cycle faster or live longer than normal cells. Cell growth at any rate within a new niche is sufficient to allow formation of a tumor. Since tumor progression or clonal evolution can generally be envisaged to involve expansion of a cell into a new microniche, cancer genes certainly do not have to directly affect cell cycle kinetics or cell death inhibition.

MORPHOLOGY AND ECOLOGY NEED TO BE CONSIDERED TO UNDERSTAND CHANGES IN FITNESS

A walk through a forest shows a virtually limitless variety of successful strategies in usual evolution. From a classical evolutionary perspective, morphologic adaptations rather than altered reproductive kinetics are more directly related to the mechanisms of altered fitness [Futuyma, 1998]. For example, modifications of hand [Susman, 1994] and brain structure [Conroy et al., 1998] are clearly more relevant to the evolution of primates than changes in fecundity or longevity per se.

Just as Darwin recognized that there are limitless numbers of mechanisms for increasing fitness [Darwin, 1859], mechanisms for clonal expansion should be considered to be practically innumerable. A few of the mechanisms independent of cell cycle and cell death kinetics per se include angiogenesis [Folkman et al.,

2000], altered relationship with the extracellular matrix [Huang and Ingber, 1999], escape from immune surveillance [Pettit et al., 2000], and resistance to chemotherapy [Shoemaker, 2000; Bredel, 2001]. It should be anticipated that many cancer genes will function entirely during interphase, rather than having a direct effect on cell cycle progression or susceptibility to apoptosis.

Evolutionary biologists further recognize that the morphologic features of a species and its precise environment must be simultaneously characterized to make reasonable hypotheses for the mechanism of an adaptive evolutionary process. For example, the evolutionary innovations in marine mammals are unlikely to be relevant to the evolution of mammals in a forested environment. Likewise, the mechanisms for clonal evolution cannot be understood without considering the cell of origin and its microenvironment, as well as the morphologic changes that accompany clonal expansion. To illustrate, even if the timing and molecular defects were known in the hypothetical carcinogenic pathway shown in Figure 1, it would still be impractical to make reasonable hypotheses for how genes *A*–*F* functioned to allow clonal expansion of populations 1–6. Cancer genes *A*–*F* could function by changing cell cycle kinetics, preventing apoptosis, altering susceptibility to immune clearance, increasing angiogenesis, altering stromal relationships, any combination of the above, or even none of the above! From a full evolutionary perspective that incorporates cell and tissue structure, it does not appear useful to reduce the countless possible mechanisms into a handful of "hallmark" [Hanahan and Weinberg, 2000] categories.

THE RELATION BETWEEN PHENOTYPIC CHANGES AND EVOLUTIONARY MECHANISMS

Although a comprehensive evolutionary framework seems to expose an unworkably large number of potential mechanisms for increasing "cellular fitness" during clonal evolution, the power of an evolutionary perspective is that it allows ecological and morphologic observations to reduce the countless possible mechanisms to a few likely and testable hypotheses when attention is restricted to particular evolutionary branches. In fact, Darwin could often deduce how hereditary elements functioned to alter fitness before the actual existence of genes was known! To illustrate, Figure 2 shows the phylogeny of Darwin's Finches, as determined after Darwin's death through careful study of their ecology and morphology early in the 1900's (reviewed in [Grant, 1999]). Recent molecular data confirms the basic structure of this phylogenetic tree

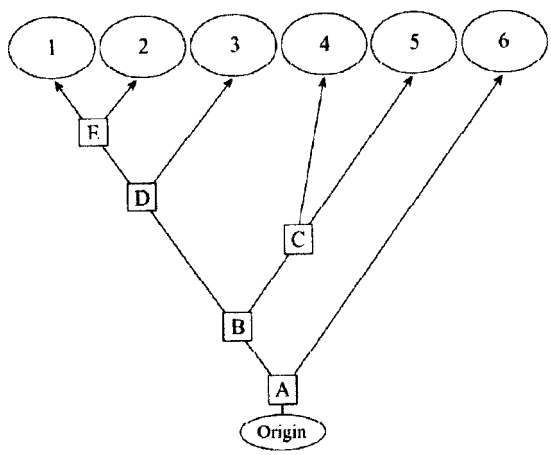

Fig. 1. A hypothetical carcinogenic evolutionary pathway is shown in which cancer genes *A*–*E* lead to clones 1–6. Without knowledge of the distinctive morphologic features of the clones or the microenvironment (niche) into which they expand, there are limitless numbers of potential mechanisms for clonal evolution.

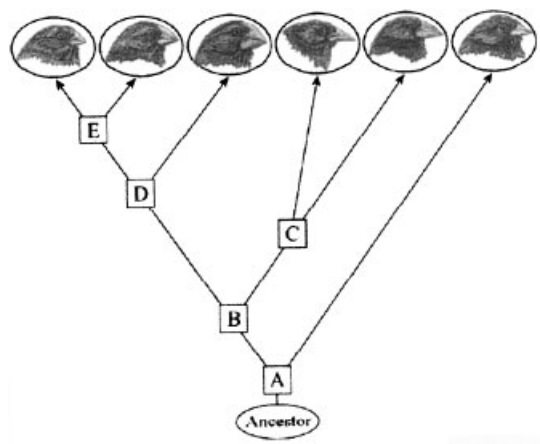

Fig. 2. When ecology and morphology are added to a phylogeny, deductions can be made about the function of the genes permitting evolution. The most accurate morphologic feature for distinguishing these Darwin's Finches is bill shape. Bill shape is genetically determined, and it appears to be the mechanism for adaptive diversification of these species. (Adapted from THE BEAK OF THE FINCH by Johnathan Weiner, 1994. Original drawings © 1994 by K. Thalia Grant. Used by permission of Alfred A. Knopf, a division of Random House, Inc.)

[Petren et al., 1999]. (However, note that molecular phylogenetic studies do not disclose the mechanisms for this adaptive speciation.) Early in *The Origin of Species*, Darwin wrote: "A corollary of the highest importance may be deduced from the foregoing remarks, namely, that the structure of every organic being is related, in the most essential yet often hidden manner, to that of all the other organic beings, with which it comes into competition..." [Darwin, 1859]. In this passage and in the following pages, Darwin "deduces" that the heritable structural characteristics distinguishing related species provide a representation of the functional changes that allow their adaptive evolutionary divergence. When adaptive speciation events have been studied in great detail, this indeed seems to be the case, as shown in Figure 2. Differences in the shape of the beak are the most characteristic features distinguishing the species in Figure 2 [Grant, 1999]. The differences in beak shape that distinguish the various species of Darwin's Finches have a genetic basis [Grant, 1999]. The differences in beak shape maximize efficient exploitation of different foods [Grant, 1999], and they appear to contribute to reproductive isolation by changing the vocal qualities of the species [Podos, 2001]. The structural features that distinguish the species in Figure 2, therefore appear to be the mechanism for adaptive speciation in this group.

Darwin's term "organic being" in the above passage encompasses cancer cells, since they are self-replicating. To develop the analogy further, each step in the development of a particular cancer is akin to adaptive speciation [Futuyma, 1998]. Pathologists' morphologic criteria for distinguishing proliferations during clonal evolution become analogous to biologists' criteria for morphologically distinguishing two closely related species. Structural features that biologists use to distinguish two closely related species (particularly species that arose through adaptive speciation) are expected to reflect in some manner the activity of the genes mediating their evolutionary divergence. It seems that Darwin would have "deduced" that cellular-level criteria of malignancy reflect the altered cellular fitness afforded by cancer genes. With 1,208 pages devoted to describing the numerous cellular-level structural features diagnostic of cancer in one textbook [DeMay, 1996] one should anticipate the existence of a large number of mechanisms for altering cellular fitness.

CELLULAR LEVEL CRITERIA OF MALIGNANCY PROVIDE INSIGHT INTO CANCER CELL PHYSIOLOGY

Genetic changes during the evolution of Darwin's Finches affect the finch beak directly. Indirect effects on the Finch ecological community are more difficult to predict. Since the unit of selection in cancer is the cell, the relation between genetic changes and the criteria of malignancy should be most direct when studying the morphologic changes in the affected cancer cell itself, rather than the associated changes in larger-scale tissue architecture.

The prediction that cellular-level criteria of malignancy often relate directly to the genetic changes in the affected cancer cells is borne out in the few studied examples. Translocations in either RET or TRK tyrosine kinases are associated with the earliest stage of development of papillary thyroid carcinoma, and in normal human thyroid cells these cancer genes induce the diagnostic nuclear envelope irregularity and chromatin clearing of papillary thyroid carcinomas [Fischer et al., 1998a, 2003][1]. RAS oncogene activations mediate a coarsening of chromatin familiar to pathologists, when expressed either in thyroid follicular cells or in fibroblasts [Fischer et al., 1998a,b]. The cancer genes SRC, FES, RAF, and MOS induce nuclear changes similar to those of RAS when expressed in fibroblasts [Fischer et al., 1998b]. Expression of p53 correlates strongly with the development of anaplastic nuclear features in some tumors, for example anaplastic thyroid carcinoma [Farid, 2001]. Amplification of Her-2/neu is correlated with high nuclear grade in breast cancer [Ho et al., 2000].

Until the early 1990's, it was not possible to counter a common notion that fixation and staining of cells creates "diagnostically useful artifacts," devoid of any deeper biologic significance. However, numerous studies using fluorescently tagged proteins [Gerlich et al., 2001] and nucleic acids [Solovei et al., 2002] in living cells have established that the fixatives and stains developed over many decades provide an accurate representation of the

[1]Fischer A.H., Greco A., Jhiang S.M., Pierotti M., Taysavang P., and Khan A. Nuclear envelope and chromatin remodeling by both RET/PTC1 and TRK-T3 depend on an Shc/FRS-2 docking site. (Manuscript in preparation.)

organization of living cells, and this is probably why they remain useful to this day.

At the levels in biology where physiology is understood best, it is obvious that structure and function accommodate each other in such a way that changes in function require an alteration in structure. For example, at the organismal level, the ability to successfully exploit different-sized seeds amongst Darwin's Finches requires a different beak structure. At the protein level, it is obvious that structural modifications are required to allow altered substrate specificity or catalytic activity. Unfortunately, at the cellular level, the relation of structure and function remain difficult to visualize. In the absence of a complete understanding of cell physiology, cell structure is often ignored in models of cancer, or it is considered somehow to be a consequence of the cells' altered functional state. Changes in cell structure accompanying carcinogenesis should generally reflect an adaptive requirement of the cell to achieve a cancer-associated physiology, in the same way that structural changes at any other level in biology are interpreted by evolutionary biologists.

Figure 3A shows an accepted phylogeny in which thyroid epithelium gives rise to follicular adenomas or papillary thyroid carcinomas [Wynford-Thomas, 1993]. By analogy to Figure 2, the altered nuclear envelope and chromatin diagnostic of papillary thyroid carcinoma (mediated by RET/PTC and TRK/PTC) should reflect the functional changes that allow clonal expansion. It may not yet be possible to know how altered large-scale nuclear envelope structure is functionally significant to papillary thyroid carcinomas [Fischer et al., 2001, 2003], but these nuclear changes provide the best available clue to the mechanism, and any hypothesis for how papillary thyroid carcinomas grow must account for the altered large-scale nuclear structure.

Diagnostic large-scale structural consequences of cancer gene activation seem to provide a more rational endpoint for tracing signaling pathways than any assumption of altered growth kinetics per se. The diagnostic nuclear features that distinguish papillary thyroid carcinoma from follicular neoplasms cannot be explained on the basis of altered cell cycle kinetics or apoptotic rates [Basolo et al., 1997]. The diagnostic chromatin coarsening induced by RAS and other cancer genes in vitro correlate closely with in vivo markers of aggressiveness, and these chromatin changes also cannot be explained on the basis of altered cell cycle kinetics [Fischer et al., 1998b].

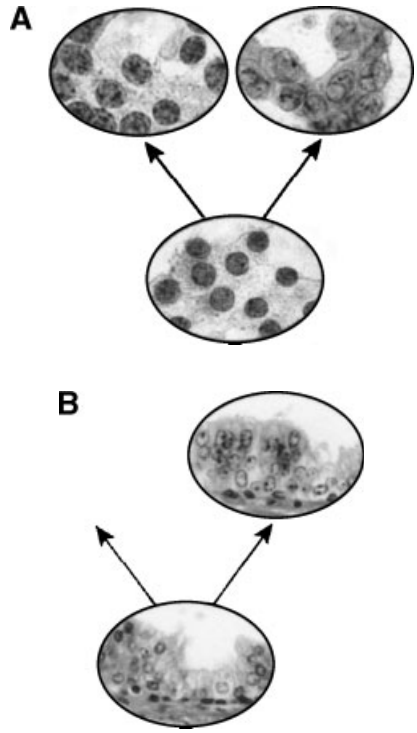

Fig. 3. Cellular-level criteria of malignancy should reflect the functional changes allowing clonal evolution, and they should be induced by the cancer genes that promoted clonal evolution. In (**A**), the cellular-level structural features that distinguish papillary thyroid carcinoma (**upper right**) from either follicular adenoma cells (**upper left**) or normal thyroid cells (origin) are dispersal of heterochromatin and nuclear envelope irregularity. These structural features have been shown to be mediated by the cancer genes active in these tumors [Fischer et al., 1998a, 2003]. **B**: Shows normal prostate epithelium giving rise to high-grade prostatic intraepithelial neoplasia (upper right) characterized by nucleolar enlargement. By analogy to Figure 2, prostate cancer genes operative at the PIN stage are predicted to induce nucleolar enlargement [Fischer et al., 2004].

Conservation of a structure through evolution is a strong indication that the structure is functionally significant [Futuyma, 1998]. For any one particular cancer, there are common cytologic changes that are conserved throughout tumor progression. For example, marked nucleolar enlargement is a useful criterion for diagnosing the earliest stage of prostate cancer development—prostatic intraepithelial neoplasia—and it persists throughout tumor progression in prostate cancer [Rosai, 1996] (Fig. 3B). The evolutionary perspective predicts that one or more early genetic events in prostate cancer development involve an alteration in nucleolar/

ribosomal metabolism, and that this alteration in nucleolar metabolism should be functionally significant [Fischer et al., 2004]. If current concepts of nucleolar metabolism cannot explain the nucleolar alterations (it seem unlikely that prostatic intra-epithelial neoplasia proliferates so rapidly that many more ribosomes are required), then there should be novel cell physiologies involving nucleoli or ribosomes [Fischer et al., 2004].

INCORPORATING TISSUE-LEVEL CRITERIA OF MALIGNANCY INTO THE EVOLUTIONARY MODEL

Although tissue-level criteria of malignancy may not relate to cancer genes as directly as cellular-level criteria, tissue-level criteria provide an essential insight into the interplay between the altered cellular fitness and the constraints imposed by the cell's microenvironment. It is feasible for pathologists to begin to propose testable hypotheses for the reciprocal changes in cancer cell function in relation to the cell's environment that can explain how clonal expansion occurred, and why tissue-level patterns are diagnostic. For example, during colon cancer development, it would be sufficient for colonic epithelial cells to maintain replication competence away from the base of the crypts onto the surface for clonal expansion to occur (Fig. 4, lowest oval). Without any other adaptive change, further increase in cell numbers of a tubular adenoma ultimately becomes limited to the border with the normal glands (regardless of how fast the cells divide), since a tissue level diagnostic trait of tubular adenoma cells is that they grow as if they required a connection to the basal lamina. Two types of changes in cellular fitness can allow cells in the midregion of the tumor to increase in number: the cells could induce an increase in basement membrane surface area (the diagnostic tissue-level change that pathologists call a villous adenoma, as shown in the middle oval of Fig. 4). Alternatively, the cells could acquire the ability to grow independent of a basement membrane attachment (the diagnostic tissue-level stage called high-grade dysplasia as shown in the upper oval of Fig. 4). Complete loss of cell polarity with free stratification of the cells away from a basement membrane can explain diagnostic tissue-level spherical cribriform spaces in adenocarcinoma in situ. The stage of invasion can be characterized as an acquired resistance to the negative impact of foreign extracellular matrix on epithelial cell survival. Such a tissue-level hypothesis of the mechanism of invasion conveys unequivocal histopathologic criteria, and is consonant with the observation that implantation of non-invasive tumor cells in foreign extracellular matrix (for example following a biopsy of high-grade dysplasia in a villous adenoma) reproducibly leads to death of the implanted cells. Apparently any one of these steps during colon cancer development can be achieved by several different cellular-level mechanisms since a variety of different cellular-level alterations can be seen at any of these stages.

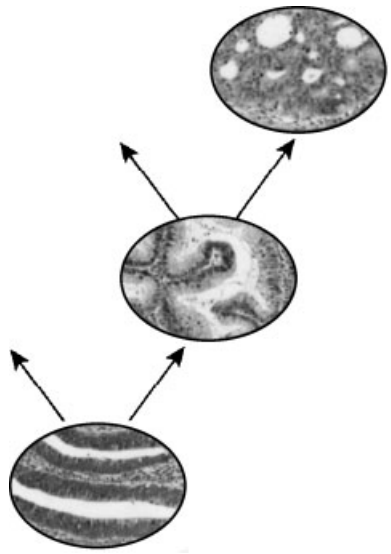

Fig. 4. Tissue-level criteria of malignancy can be phrased in terms of a hypothesis for the functional adaptations that allow clonal expansion. The mechanism for clonal expansion giving rise to tubular adenomas (**lower left**) from normal colonic epithelium appears to be maintenance of replication competence through the full height of the crypt. Tubular adenoma cells still require contact with the basal lamina. Retention of replication competence throughout the full height of the crypt permits tubular adenomas to grow only at the leading edge of the contiguous mass of cells. An increase in the surface area of basal lamina, the stage called ''villous adenoma'' (**middle figure**) would allow further clonal expansion. The ability to resist anoikis, or grow without an attachment to the basal lamina, can permit further clonal expansion in the stage termed high-grade dysplasia (**upper figure**).

Phrasing the tissue-level criteria of malignancy in terms of a likely and testable hypothesis for the mechanism of the altered fitness should add reproducibility to cancer diagnosis beyond what is possible with pure pattern recognition approaches to diagnosis, and this

approach appears useful for teaching cancer diagnosis.

WHY ARE THERE SO MANY OVERLAPPING CRITERIA OF MALIGNANCY, AND WHY ARE THEY IMPERFECT?

From the evolutionary framework, the numerous types of human cancers are characterized by numerous tissue-level or cellular-level criteria of malignancy (even when arising from the same cell of origin), presumably because they acquire different types of fitness alterations. The existence of so many varied forms of pre-invasive breast epithelial proliferations predicts the existence of a wide variety of trophic influences on mammary epithelial cells. Conversely, the similarity of many poorly differentiated tumors can be explained by the fact that similar sets of genetic alterations are known to contribute to the malignant phenotype of cancers starting from diverse origins. Normal cells can sometimes share cytologic features with cancer cells, presumably because normal cells sometimes require the activation of cellular oncogenes, and therefore they may at times share functional alterations with cancer cells.

Cell structure, as evident in conventional fixed and stained preparations, does not necessarily need to change for cell function to change. Some tumor cells (for example some types of breast cancer) show little or no alteration in cell structure at the light-microscope level. Likewise, it is clear that not all cancer genes induce perceptible large-scale changes in cellular structure (for example MAPKK in fibroblasts [Fischer et al., 1998b], and PTEN in endometrial glands [Mutter et al., 2001]). The absence of apparent cellular-level structural changes in some steps in cancer development is not evidence against the proposed evolutionary framework. When cell structural changes are not evident during a step in clonal evolution, it is probably because the commonly used histologic stains cannot disclose the altered structure that must accommodate any altered cellular function. Just as an evolutionary biologist would be severely handicapped in trying to understand a mechanism of speciation without knowing the morphologic features that distinguish two species, the absence of apparent cellular-level structural changes accompanying certain steps in cancer development, or certain cancer gene activations, is a severe impediment for developing hypotheses for the functional alterations during clonal evolution. The cancer genes that are known to induce diagnostic cell structural changes appear far easier to study.

Structural features characteristic of one evolutionary clade may be lost or modified in subsequent evolutionary steps. Likewise, cellular features may change during tumor progression when new cancer genes modify cellular structure and function.

AN EVOLUTIONARY FRAMEWORK FOR TUMOR CLASSIFICATION

A major limitation of the current histogenetic classification is that it fails to account for the molecular pathway taken by the evolving cancer. Many different molecular pathways exist downstream of any one particular cell of origin; moreover, the same molecular pathway may lead to cancer starting from many different cells of origin. The molecular pathway is expected to be important for designing anti-cancer treatments, yet for many cancers there remains a practical correlation between the histogenesis and the natural history of the tumor, or its response to chemotherapy. From an evolutionary perspective, it is clear that both histogenesis and the molecular defects in a cancer cell have adaptive significance and are therefore, relevant to classification: the cell of origin of a cancer and its microenvironment put constraints on the types of genetic mechanisms that can increase cellular fitness.

The most comprehensive model of carcinogenesis entails interpreting the molecular and morphologic characteristics of tumor cells in an evolutionary framework. A comprehensive evolutionary model of cancer development that incorporates the criteria of malignancy provides the best available representation of both the constraints on normal cell growth and the specific cellular mechanisms operative in cancer. If we had a better representation of the constraints on normal cell growth and the functional changes that occur during cancer development, then the criteria of malignancy would not have remained useful to the present day. The task at hand is to begin to describe a precise structural basis and functional significance for any of the various criteria of malignancy. This will require restricting attention to particular steps in clonal evolution, and study-

ing cell physiology in the appropriate native microenvironment.

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