
Clonality Studies in the Analysis of Adrenal Medullary Proliferations: Application Principles and Limitations

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Abstract

Clonality remains as the hallmark of neoplasms. A dual genetic approach using markers nonrelated (e.g., X-chromosome inactivation assays) and related to the malignant transformation (such as loss of heterozygosity analyses of tumor-suppressor genes) would provide useful clonality information from early and advanced tumor stages, respectively. Tumor progression and clonal selection would result in genetic instability and heterogeneous expression of those molecular markers related to the malignant pathway. Therefore, only the coexistence of multiple genetic abnormalities would support the clonal nature as an expression of convergent cell selection. Considering those facts, the currently available evidence on tumorigenesis and clonality in the adrenal medulla can be summarized as follows:

1. Multistep tumorigenesis defines the evolution of pheochromocytomas, as evidenced by the presence of several genetic alterations.
2. Both the significant association of nonrandom genetic alterations (specially 1p and 22q interstitial deletions) and the topographic accumulation of genetic deletions at the peripheral tumor compartment support a convergent clone selection for these neoplasms.
3. Although many genetic loci show nonrandom abnormalities, the most frequently involved locates on chromosome 1p regardless of genetic tumor background (sporadic or inherited predisposition).
4. Most pheochromocytomas should begin as monoclonal proliferations that do not always correlate with histopathologic features, particularly in inherited tumor syndromes.
5. Early histopathologic stages, described as adrenal medullary hyperplasias, are defined by hyperproliferative features in animal models and monoclonal patterns in the adrenal nodules from patients with MEN-2a.

Key Words: Pheochromocytoma; adrenal medullary hyperplasia; clonality; X-chromosome inactivation; loss of heterozygosity; clone selection; multistep carcinogenesis.

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Introduction

Although many molecular features have been described in order to characterize neoplasms better, its definition is still descriptive. All these features try to distinguish neoplasms from nontumoral conditions and malignant from benign neoplasms as well. Malignant tumors (specially carcino-

mas) evolve through continuous and additive changes, both at genetic and phenotypic levels, ending in the complete malignant phenotype. The most widely accepted theory proposes a clonal origin for them, in contrast to the heterogeneous composition in most normal tissues [1,2]. Therefore, monoclonal cell populations

arisen from heterogeneous normal tissues strongly suggest a neoplastic nature. Different and variable success has been reported for several markers, but clonality still remains as the hallmark of neoplasms. Before any specific reference to clonality in pheochromocytomas and related lesions of the adrenal medulla, some general biologic considerations would help us to understand the strength and weakness of molecular techniques in the analysis of clonality. Those considerations will focus on the relationship among cellular kinetics, neoplastic transformation, and clonality. A brief review of the morphologic criteria of pheochromocytomas (PCC) and adrenal medullary hyperplasia will follow, and finally, the current information on clonality in the analysis of adrenal medullary proliferations will be covered.

General Considerations on Malignant Transformation and Clonality

The term clonality has been broadly used, and it is related to the presence of cellular clones in tissues, although that terminology can be applied at different organizational levels. Those clones represent the groups of genetically identical copies of a single progenitor. The strict application of that definition would require the sequence of all DNA in order to exclude any polymorphism, but that approach is not viable. Therefore, different markers have been proposed to obtain such information using more or less complicated and simple techniques. In any case, the presence of groups of genetically identical cells must be related to proliferative advantages of those cells over the remaining. For that reason, a close relationship can be drawn between clonality and kinetics features. The expression of

those features is variable from the conventional histologic level to the molecular one.

The current definition of neoplasm is descriptive and closely related to clonality. Neoplasms are defined as abnormal and self-maintained proliferations, resulting in the presence of new tissues. Those new tissues should come from a group of cells sharing a particular and common advantage. Therefore, a clear relationship between both concepts (neoplasm and clone) is present. Its application to everyday practice of pathology would also contribute to explaining the development of diagnostic criteria of neoplasm in many areas of surgical pathology. Just as an example, the diagnosis of malignant lymphomas, especially at the extranodal level, requires the presence of a monotonous proliferation of lymphoid cells that substitutes the normal tissue architecture and results in a mass. Those criteria represent, at the histologic level, the morphologic evidence of an expansive growth of neoplastic cells. Sometimes, those cellular expansions share specific phenotypes (cytoplasmic or surface markers, and so forth), which allow their identification. Such an approach would require quantification of its expression in order to distinguish the abnormal proliferation from its normal counterpart, e.g., light-chain restriction of immunoglobulin in lymphoid neoplasms or the flow cytometric analysis of surface lymphoid markers in hematologic malignancies.

On the other hand, the neoplastic transformation is a multistep process, resulting in a complex mixture of tissues with heterogeneous genetic background [1]. The results of every molecular test will depend on the timing between the evaluated biologic feature and the specific marker (Fig. 1). Simultaneously, the sensitivity and specificity of each molecular marker used to test any biologic feature, such as clonality,

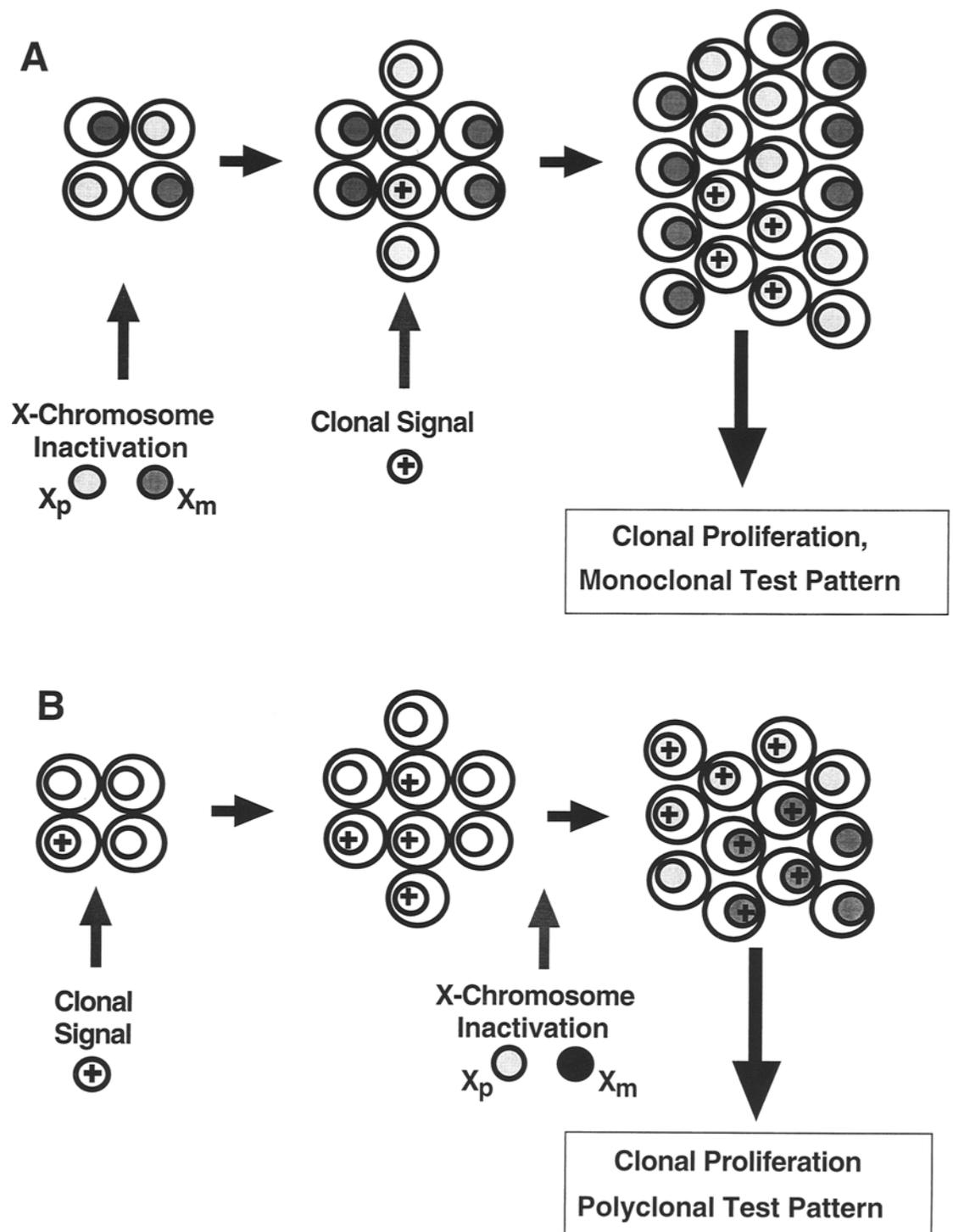


Fig. 1. Timing between the clonal signal stimulus and the X-chromosome inactivation (XCI). The interpretation of any test result (in this case XCI) will depend on the temporal relationship between the marker used (methylation of inactive alleles on X chromosome) and the biologic feature evaluated (in this case clonality). Normally XCI takes place early before any clonal signal (labelled + in the figure), ending in monoclonal patterns (**A**). Those situations where the clonal signal precedes the XCI would eventually result in polyclonal patterns, even for monoclonal proliferations ("pseudo-polyclonal", **B**).

should be checked with samples of different and known genetic constitution. In addition, those molecular markers of clonality should be evaluated in relation to two main features, cellular kinetics and the neoplastic pathway. Any marker related to the malignant pathway will provide positive information only if that target participates in the molecular pathway of a given tumor. The current evidence suggests that malignant tumors result from the accumulation of multiple genetic events rather than from any specific sequence of genetic changes [3–5]. Some mutations are more important than others, because they facilitate additional phenotypic or genotypic changes. This reason explains certain trends in the order of mutation production, although significant variation for them is the role [6]. The lack of such a sequence and the unpredictability of genetic changes in malignancies preclude the extensive clinical use of those genetic markers with diagnostic and prognostic purposes. Finally, the timing between the clonal expansion and the presence and expression of malignant features is unknown. All these limitations should be considered to achieve reliable and useful information from any marker.

The final expression of any neoplasm depends on the progressive and convergent selection of cell populations, but several factors should be taken into consideration. On one hand, that selection will determine tumor progression and cellular heterogeneity. On the other hand, cellular selection is the expression of cell kinetics [7]. Considered from the genetic point of view, neoplasms can be characterized by multiple genetic aberrations, from gross to point mutations. However, those genetic aberrations should be “fixed” on the transformed cell before ending in a fully established malignancy. Any nonlethal genetic aberration will be “cell-fixed” if

both its transmission to descendant cells is warranted and it is able to bypass the cell-repairing systems. Otherwise, any genetic alteration leading to cellular aging, differentiation, or activation of the apoptotic pathway will be nontumor-productive (Fig. 2). This process determines a complex network where a potential genetic marker will be useful when associated with kinetic advantages responsible for cellular outgrowths.

It is out of the scope of this article to provide detailed descriptions of those markers used for testing both clonality and cell kinetic [7–9]. Essentially, two types of molecular markers provide reliable results on clonality, classified according to its chromosomal location in X-linked and non-X-linked assays. Those markers independent of the malignant transformation pathway provide more general information, especially from the early neoplastic stages. Among them, the assays based on the random inactivation of one X-chromosome in females are the most extensively used to test clonality [9]. These tests target the differential expression of alleles of X-chromosome-linked genes (mRNA or protein analyses) or the differential DNA methylation on active (nonmethylated) and inactive (methylated) alleles. Currently, the most productive marker of this sort is the human androgen receptor gene (HUMARA) owing to its high percentage of heterozygosity in the general population (around 90%) related to the presence of a highly polymorphic trinucleotide repeat region. That polymorphic region also shows a predictable methylation pattern on sequences recognized by methylation-sensitive restriction endonucleases [10], allowing allele differentiation. In fact, the information on clonality provided by both X-linked and non-X-linked assays is complementary and nonmutually exclusive, because the results from X-linked

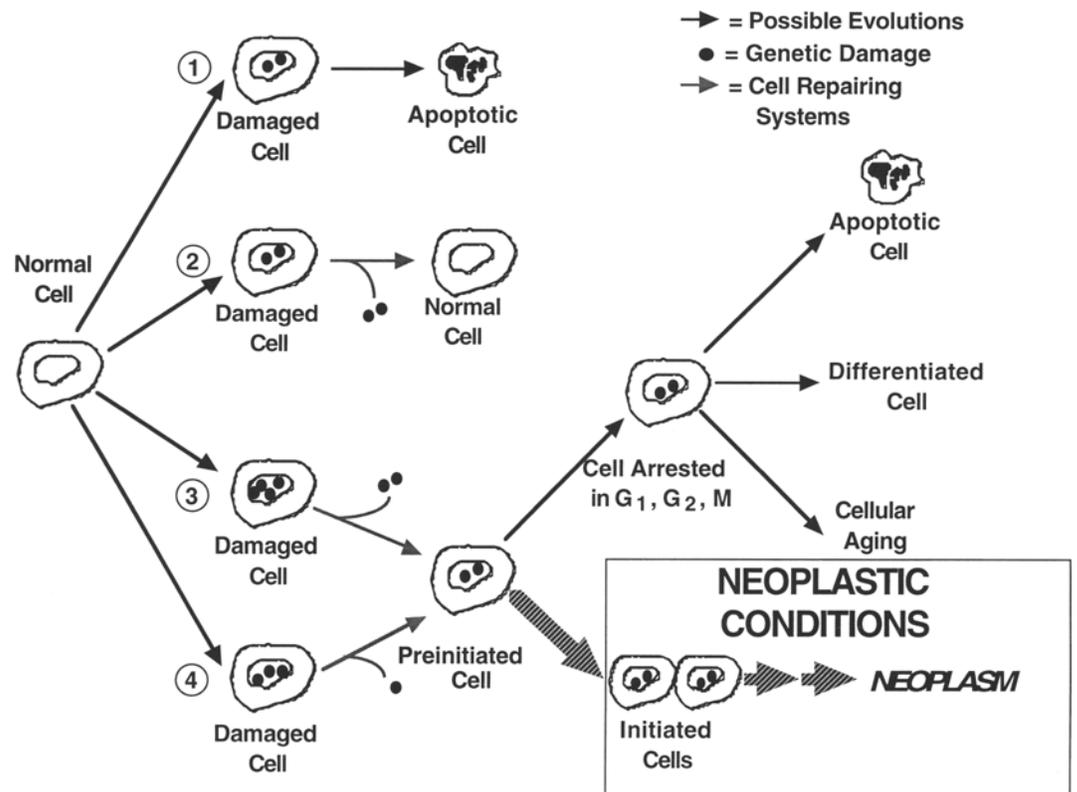


Fig. 2. DNA damage and cellular outcome. Currently, neoplasms can be considered as genetic diseases (resulted from genetic damages), which become clinically detectable after clone selection and cellular proliferation. The induction of any neoplasm requires special features for the genetic events involved in the malignant transformation. Those events (black dots) have to be resistant to the cell-repairing systems (dotted arrows), accumulated cooperatively in the cells, efficiently transmitted to descendant cells, and nonlethal (that is able to induce apoptosis). The clonal expansion of genetically damaged cells under those conditions will result in a neoplastic growth.

assays are based on genetic features usually nonrelated to the malignant transformation.

The inactivation of one X-chromosome in females is an early event during embryogenesis, described in embryos of 16–30 cells [11]. Two main consequences can be drawn, with both biologic and technical implications. First, some tissues would show the same allele inactivated and a monoclonal pattern, avoiding any positive conclusion from a tumor arisen on it (noninformative case) [2,7]. This pattern is variable from patient to patient and even from tissue to tissue in the same patient. For that reason, the so-called Lyonization ratio, reliable and appropriate results for any tumor or precancerous tissue samples

must require embryologically-related controls [12,13]. Second, most of somatic genetic alterations described in neoplasms will take place on cells showing one X-chromosome inactivated. Those genetic events associated with kinetic advantages and cellular overgrowths will probably determine a monoclonal methylation pattern of the X-chromosome if they trigger one cell or a small group of neighboring cells (Fig. 1).

Those tests based on other somatic markers potentially involved in the malignant pathway give information on the transformation mechanism. Any nonrandom genetic alteration (X-linked or not) present in most of the tumor cells will provide useful information on clonal expansion.

sion, even those as small as point mutations. Those point mutations, regardless of their location on exons or introns (the latter, also referred to as single nucleotide polymorphism, has no functional expression at mRNA and protein levels), would be related to kinetic advantages and, therefore, have been proven useful to test clonal expansions in tumors. However, only those tumors expressing that particular marker will be suitable for that analysis. Additionally, the ratio of positive results will be necessarily related with both time-independent and time-dependent features of each tumor, e.g., histologic type, location, grade, or stage. Those variables can be "confusing factors" if they are not adequately evaluated. Owing to the absence of an established timing for a particular genetic event in the transformation pathway, variably long gaps of test negativity would be demonstrated for a particular molecular marker in a given tumor (Fig. 3). That would cover the time between tumor initiation and assay conversion, giving inconclusive results. There will not be any gap time for such conversion if the tested molecular marker represents the first mutation event in the natural history of a given neoplasm. In that case, the test results would be identical to those provided by markers unrelated to the malignant transformation pathway, e.g., X-chromosome-inactivation assays. In conclusion, only multifactorial approaches considering different markers simultaneously would appropriately provide clonality information in tumor conditions.

Pheochromocytomas and Adrenal Medullary Hyperplasias: Basic Pathology

In general, the morphologic features of endocrine tumors barely correlate with biologic behavior. Many clinicopathologic

studies make the point that cytologic features, such as nuclear pleomorphism, hyperchromatism, or increased nuclear/cytoplasmic ratios, are not necessarily related with aggressive outcome. Sometime, only the presence of metastatic tumor growths enable us to establish a malignant diagnosis [14]. Therefore, in addition, histopathological studies are required to improve both diagnosis and prognosis of these neoplasms. Other approaches trying to detect the tumor at early steps (precancerous conditions or incipient neoplasms) go in a parallel direction to improve patient prognosis. Pheochromocytomas (PCC) fulfill all these general rules.

The histopathologic differentiation of hyperplastic and neoplastic conditions in the adrenal medulla is mainly arbitrary [14,15]. The first differentiating criterion is the increase in the adrenal weight, as an expression of the increased number of chromaffin cells, with expansion of the medullary compartment into areas of the gland where it is not normally present. Morphologically, hyperplastic conditions have been classified according to their growth pattern as diffuse or nodular [16]. Additionally, no specific and consistent criteria will distinguish big nodules within a multinodular background (hyperplastic condition) from small PCC (neoplastic condition). Some reports propose the nodule size as the main differentiating criterion and 1 cm as the threshold [17]. That size has been suggested as the criterion because it is the detection limit of most image diagnostic systems and represents the smallest size clinically detectable. That tumor size was also selected because the smallest PCC in the first series from the Armed Forces Institute of Pathology had 1-cm diameter [15]. No biologic reasons are provided for that distinction. On the other hand, the knowledge available for adrenal medullary hyperplasia comes from inherited condi-

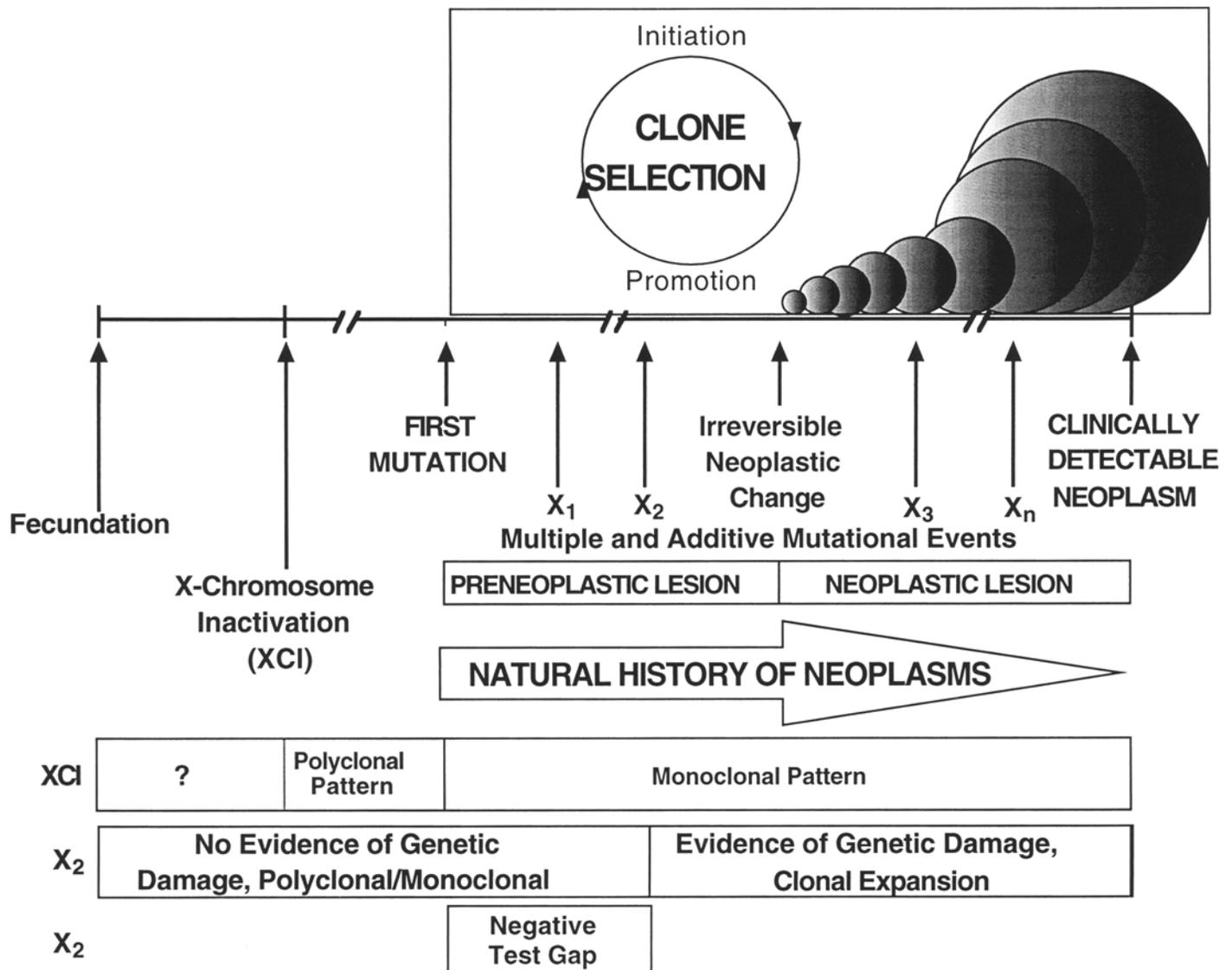


Fig. 3. Natural history of neoplasms based on clone selection and tumor progression. The natural history of neoplasms is a linear temporal expression of the convergent and endless selection process of cell clones in tumors. The clone selection results from the interaction between genetic changes (mutational events) and individual susceptibility. Progressive and cooperative changes should accumulate in cells before they are expressing a fully neoplastic phenotype. The point of irreversibility in that sequence also separates the chronologic evolution from preneoplastic into neoplastic conditions. On that background, the clonality markers will give useful information to depict the neoplastic natural history. Any particular marker will give information concerning clonality if it is involved in the malignant transformation process. Likewise, it will be concordant with XCI assays only if it represents the first mutational genetic event. All other situations will determine negative results for the marker even for already clonal proliferations (negative test gap).

tions, especially those related to MEN-2, where quantitative analyses demonstrated utility to distinguish medullary hyperplasias from normal adrenal medulla [16]. Cytologic and architectural features have showed no significant and reliable differences useful for that task [15].

PCCs have been described as sporadic neoplasms or associated to other tumors as part of inherited cancer syndromes, mainly multiple endocrine neoplasia type 2 (MEN-2), neurofibromatosis, or von Hippel-Lindau disease [18]. Clinically and histopathologically, the situation is differ-

ent for both conditions [15]. The absence of either a known genetic background or a reliable screening method precludes the systematic detection of early sporadic tumors. Therefore, most of those sporadic tumors are detected in more advanced stages, although only about 10% shows malignant behavior. Additionally, no preneoplastic changes are usually associated with them. The opposite situation characterizes the inherited PCC: they are usually detected earlier owing to genetic and clinical screening, and medullary hyperplasia, either diffuse or nodular, is also present.

Clinicopathologic studies mainly define benign and malignant PCCs. Currently, a bona fide diagnosis of malignant PCC requires the demonstration of metastases, defined by the presence of tumor overgrowths in sites where chromaffin tissue is not normally present [15]. Those organs include lymph nodes, liver, and bone [14]. The presence of nonneoplastic chromaffin tissue in other organs (as a physiologic finding or as a part of hyperplastic conditions) avoids the establishment of malignant diagnoses based merely on the histopathologic evidence of such tissue. The demonstration of truly metastatic outgrowths would require additional studies in order to prove clonal identity in both primary and metastatic tumor [19]. Relatively clear patterns are usually shown at both ends of the pathologic spectrum (benign and malignant tumors) [20]. A slightly different situation would be expected in borderline conditions, such as locally invasive tumors [21,22]. Those tumors are characterized by variable invasion of perirenal soft tissue, with no evidence of distant metastatic growths. Its association with lymph node metastases needs to be defined and the long-term patient outcome is unknown [15]. Other parameters are required to obtain a full understanding on the biologic behavior of these particular tumors.

Clonality Analyses of Adrenal Medullary Proliferations

Genetic analyses of different molecular markers, especially those of polymorphic DNA regions, have provided valuable information on the pathogenesis of endocrine neoplasms, both sporadic and familial. Those polymorphic DNA areas allow reliable allele identification, and, therefore, DNA loss would be expressed as reduction to hemizyosity. According to Knudson's hypothesis, DNA loss would explain neoplastic development and progression if tumor-suppressor genes are involved [23,24]. A relatively common pattern for most PCCs came from those studies, showing genetic deletions (demonstrated by loss-of-heterozygosity [LOH] analyses) at several loci, particularly at 1p, 3p, 11p, 11q, 17p, 17q, and 22q [25–31]. Those interstitial DNA deletions mainly involve both known tumor suppressor genes, such as von Hippel-Lindau gene locus (3p25) [30], neurofibromatosis 1 locus (17p) [25], or p53 locus (17p) [26], and unknown tumor-suppressor genes at 1p (at 34–36, distal to D1S73 and proximal to D1S63), 11p, 11q, and 22q (distal to D22S24 and proximal to D22S1) [29–31]. Additionally, activating point mutations of genes involved in other endocrine tumors have been demonstrated in both sporadic and familial PCCs, including ret proto-oncogene [32] and the G-protein gene encoding for Gs α (gsp) [19].

Evidences of Clonality Based on X-Chromosome Inactivation Analysis

Preliminary results revealed monoclonal methylation pattern in the alleles of the androgen receptor gene (HUMARA assay of clonality) in 87% of informative cases from a series of sporadic PCCs [22]. After microdissection, at least two different

samples were analyzed in each neoplasm, from the peripheral and the internal tumor areas, respectively. Both tumor areas provided concordant methylation patterns, consistent with a monoclonal origin for this series of cases. The remaining 13% of informative PCCs showed balanced methylation of both HUMARA alleles, pointing to a polyclonal tumor growth. The latter group of cases corresponded to locally invasive PCCs (as defined by periadrenal soft tissue invasion) with high cell turnover rate (both MIB-1 labeling index and *in situ* end-labeling index received the highest scores for this series) [21]. Histologically, stromal overgrowth with a prominent smooth muscle differentiation (positive immunoeexpression of smooth muscle type actin and desmin) was revealed by dissecting the neoplastic cell nests. Differential microdissection from several tumor locations always confirmed the initial results. Excluding the possibility of significant contamination with host normal cells by careful and repeated sampling with microdissection, those findings could be the result of (1) true polyclonal tumors, which, because of an unfinished process of neoplastic cell selection, still show coexistent cell clones descended from different progenitor, or (2) an abnormal methylation of the HUMARA locus during tumor progression (hypermethylation of the active allele) expressing pseudopolyclonal patterns in true monoclonal neoplasms.

Another method used to investigate clonality, also based on the X-chromosome inactivation, depends on the mosaicism of protein expression in the normal tissues from heterozygous women showing two isoforms of the enzyme glucose-6-phosphate dehydrogenase (G6PD) [33–35]. The original report of clonality in medullary thyroid carcinomas and PCCs associated with multiple endocrine neoplasia

relies on this technique [33,34]. The authors reported the presence of only one isoenzyme in tumor tissue from patients proven to be heterozygous for that marker and concluded that the initial mutation produces multiple clones of defective cells. Thereafter, each tumor arises as a final mutation in one clone of these cells. Essentially, the proposed hypothesis claims for an initial polyclonal and nonselected proliferation of defective cells, which then turns monoclonal through a process of cellular selection [36]. The histopathologic features in each stage have not been established, although theoretically adrenal medullary hyperplasia and PCC would represent the preneoplastic and neoplastic conditions, respectively.

Following the same methodology described for sporadic PCCs, HUMARA clonality assay was also performed with PCCs from members of an MEN-2a family [22]. The patients developed PCCs with no special histologic features, arisen on multinodular medullary hyperplasias. All except one tumor nodule revealed monoclonal methylation patterns, regardless of their sizes. The internodular adrenal medulla was required to show polyclonal methylation pattern in order to consider the case informative (criteria of case inclusion). Therefore, no conclusions could be obtained from the adrenal medulla with diffuse growth pattern. These results support the multistep tumorigenesis in the adrenal medulla of patients with MEN-2a [16,17,26]. An initial polyclonal stage would progressively evolve into monoclonal cell growths as the result of clone selection [6,36]. On the other hand, they also question the validity of nodule size as diagnostic criteria to distinguish hyperplasias from neoplasms. Tumor (or nodule) size is a time-dependent parameter and revealed a low specificity for case stratification by clonal pattern. That finding can-

not be considered surprising for neoplastic processes with early transformation events, as those reported in most inherited tumor syndromes [23,24].

Evidence of Clonality Based on Tumor Genetic Alterations and Multistep Tumorigenesis

Although it is generally accepted the clonal evolution takes place in neoplastic transformation [1,6], some clonality studies have reported controversial results in early neoplasms and preneoplastic conditions of endocrine organs [37]. Several studies essentially confirm the multistep hypothesis for PCC tumorigenesis. Although the genetic mechanism of tumorigenesis has been referred to as different in pheochromocytomas and extra-adrenal paragangliomas [30], the same genetic targets seem to be involved in both sporadic and familial PCCs [26,29]. The general considerations mentioned above to analyze the malignant transformation and clonality [7] lead us to conclude that all these reports only provide information on clonal expansions in pheochromocytomas. The genetic homogeneity for a given marker would point to a kinetic advantage, provided by the marker itself or linked to it, that represents the basic mechanism of cell selection and tumor progression [1,6]. However, this isolated finding does not prove that all tumor cells come from the same progenitor and they are truly monoclonal. The same mutagenic event can affect cells from different progenitors (Fig. 4). Therefore, the descendant cells would share the same genetic abnormality (homogeneous pattern for a particular molecular marker, as expression of its clonal advantage and expansion), although they may have distinctive genetic background (different progenitors and true polyclonal origin).

Several tumor-suppressor genes have been demonstrated to play an important role in the above-mentioned clonal expansions of adrenal medullary neoplasms. As previously shown for other neoplasms, the tumor initiation and/or progression in PCCs involves multiple genes, mainly located by LOH analyses on chromosomes 1p, 3p, 17p, and 22q [26,29-31]. Some of these markers have also revealed significant association with clinicopathologic parameters, such as tumor volume (in the case of DNA deletions located on 1p, 3p, and 17p) [26] or with a distinctive transformation pathway (i.e., 1p34-36 and 3p25 deletions have been found in 45 and 56%, respectively, of PCCs, but not in extra-adrenal paragangliomas, whereas 3p21 deletions have been described in 50% of extra-adrenal paragangliomas, but not in PCCs) [30]. The relative incidence of each genetic alteration is quite variable from series to series, probably in relation to the limited number of cases analyzed. However, all series agree to show 1p deletions as the most frequent genetic finding, although no reference is available about its timing compared with other genetic abnormalities. That information would be especially valuable for the comparison between PCCs and adrenal medullary hyperplasias in order to determine its real nature either as an initiation-related genetic event or as a consequence of tumor progression.

On the other hand, the inherent genetic instability associated with neoplasms would explain the coexistence of several genetic abnormalities [1,6]. In that way, a significant association between interstitial deletions on 22q and 1p has been reported [29]. That finding suggests that the inactivation of multiple tumor-suppressor genes is required for PCCs development and progression (multistep tumorigenesis).

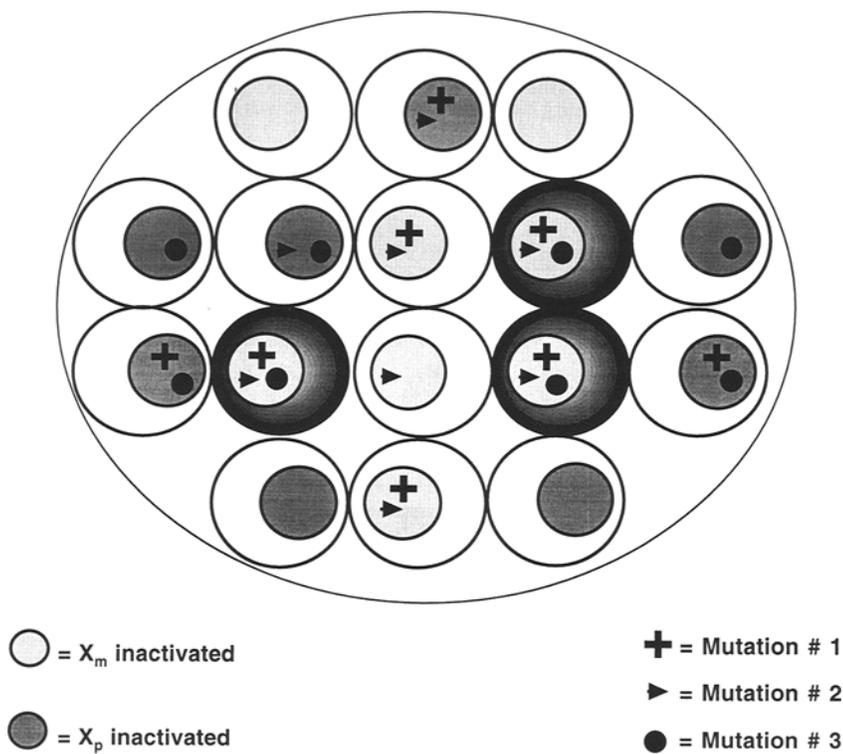


Fig. 4. Genetic alterations and clonality. LOH analyses will provide clonality information only if several markers are used. Otherwise, the presence of LOH in a given cell population only informs about clonal expansion.

Considering tumor heterogeneity, an additional conclusion arises from that association. From the statistical point of view, homogeneous genetic alterations would show a low probability for the random association of two or more molecular markers in tumor cell populations from different origins (polyclonal tissues). Previous reports on LOH analyses, using Southern blot hybridization [38] and PCR-based techniques [39], have shown random and nontumor-related DNA deletions in 4–20% of normal tissues [40–42]. These percentages should be considered from two perspectives: (1) as a potential “confusing factor” in the analysis of case series, and (2) as the limiting threshold in order to interpret appropriately the biologic significance of any association between DNA deletions and tumor conditions. Heterogeneity is evident among histologically

similar neoplasms from different patients (intertumor heterogeneity) and among different cells of the same neoplasm at a single time (intratumor heterogeneity), as well as at different points in time (tumor progression). Considering that variability, intratumor heterogeneity for LOH of multiple gene loci can be exploited as biparametric markers for the analysis of clonal selection in tumor progression that LOH genicity can be expression of either selective tumor evolution or simple passive byproduct of other mechanisms, such as genetic instability. The association of multiple genetic alterations would become statistically less probable as the number of molecular markers increases [43]. Then the random association of those markers would be better explained by a convergent selective process ending in the presence of a dominant clone [1,6]. That selection mechanism of cell clones with relatively homogeneous genetic constitution would represent the best approach currently available to define clonality based on tumor genetic markers. From this point of view, clonality could be considered the key element to understanding the endless biologic process of initiation and progression of tumors (cause and consequence at the same time) that also determines the otherwise linear natural history of neoplasms (Fig. 3).

Some experimental results (personal observations) support this point of view, based on the analysis of five polymorphic DNA regions (microsatellites) located on introns of four tumor-suppressor genes (p53, Rb, WT1, and NF1). Heterogeneous DNA loss of wild-type alleles (LOH analyses) was revealed in informative sporadic PCCs, involving p53 in 45%, Rb in 25%, WT1 in 44%, and NF1 in 50%. The comparative study of peripheral and internal tumor areas confirmed an increased accumulation of genetic deletions at the peripheral tumor compartment, expression of

both tumor progression, and multistep tumorigenesis. At that peripheral level, two or more genes showed LOH in agreement with the genetic instability of neoplasms. The low probability for random and simultaneous DNA deletion in nontransformed cell populations point to clone selection, ending in outgrowths of the "selected" clone, which becomes dominant and so-called monoclonal. The final tumor picture as monoclonal proliferation thus results from a selective process on a genetically heterogeneous cell population (Fig. 4).

Other factors also contribute to neoplastic development in endocrine organs. Somatic mutations of G-protein genes result in the constitutive activation of G-proteins and in an overall increase of the endocrine function. Along with the functional enhancement, proliferative advantages related to the trophic effect associated with the hormonal action have also been reported in these conditions [19]. That reason would contribute to explain the frequent association between overgrowth and hyperfunction of endocrine organs. G-proteins represent a key element of the intracellular signal transduction linking the extracellular ligands and the final cellular response. The active signal transduction normally present in most functional endocrine systems would explain the high sensitivity of those organs to abnormalities in that central pathway of signal transduction. That proliferative advantage would play an important role in the kinetic evolution and progressive selection resulting in cellular transformation in those disorders. Activating mutations at codon 201 of *gsp* have been found in different endocrine disorders, both neoplastic (pituitary adenomas, follicular thyroid adenomas, parathyroid adenomas, chemodectomas) and nonneoplastic (parathyroid or adrenal cortical hyperplasias) [19]. The same activating mutation has also been reported in primary

and metastatic PCCs as well as in extra-adrenal paraganglioma from patients found to be wild-type at the germline DNA (extracted from leukocytes). That presence of concordant G-protein gene mutations in such a wide range of endocrine conditions and in different endocrine disorders in the same patient is consistent with a common underlying etiology.

Tumor Susceptibility and Clonality in PCC of Inherited Cancer Syndromes

The basic molecular mechanism is the same for both sporadic and inherited PCCs. However, the genetic background provides some valuable insights to understanding the general transformation process and the clonal evolution of tumors. The inherited cancer syndromes are essentially characterized by germline genetic abnormalities in certain DNA targets, frequently point mutations. Those abnormalities used to be activating point mutations targeting proto-oncogenes, such as *ret* in MEN-2 syndromes [32], or inactivating point mutations on tumor-suppressor genes, e.g. von Hippel-Lindau disease [18,28] or neurofibromatosis 1 [25]. In both cases (activating or inactivating mutations), the basic mechanism for the clonal expansion is the proliferative advantage provided by an appropriate genetic network (usually as additional somatic alterations on different genes) along with the increased susceptibility to neoplastic transformation (related with the germline mutation). Although a similar genetic background defines a given syndrome, different transformation pathways have been described in association with each tumor type and location. Therefore, variable relative frequencies of each genetic marker have been reported, i.e., using restriction fragment-length polymorphism analysis, 1p LOH has been found in all

PCCs associated with MEN-2 syndromes (67% including sporadic and Von Hippel-Lindau PCCs), whereas it was detected in only 13% of MEN-2-related medullary thyroid carcinomas [26]. The germline abnormality provides an increased susceptibility for tumor development that modulated by the cellular environment, will only end in neoplasms if multiple genetic loci are also involved (multistep tumorigenesis). Regardless of the predisposing genetic factor, chromosome 1p deletions are again the most frequently found molecular markers, as mentioned for sporadic PCCs (*see Evidence of Clonality Based on Tumor Genetic Alterations and Multistep Tumorigenesis*) [26,29,31].

The genetic background also modulates and determines the molecular mechanism involved in the transformation process. The most frequent inactivation mechanism of tumor-suppressor genes in PCCs involves both an inactivating point mutation in one allele (missense or nonsense mutations usually present as a germline defect) and the loss of the wild-type allele [26,27], analogous with the model proposed for different inherited malignant tumors [23,24,41,42,44,45]. However, additional inactivating mechanisms should be considered when no DNA loss of the wild-type allele is revealed. Such mechanisms include intragenic somatic mutations in the wild-type allele (also called homozygous inactivation) and hypermethylation of the tumor-suppressor gene as described for von Hippel-Lindau gene [28,46,47]. On the other edge of the spectrum, several activating mutations in the *ret* proto-oncogene characterize different types of MEN-2 syndromes and familial medullary thyroid carcinomas (FMTC) [48]. Recently, a mutation of *ret* codon 768 in exon 13 was found to segregate with the FMTC phenotype (multiple cases of medullary thyroid carcinomas or C-cell hyperplasia),

but not with the adrenal medullary hyperplasia in a large multigenerational family [32]. Those preliminary data should provide information on additional factors responsible for the development of specific tumor types. However, these results should be taken with caution because of the short number of cases analyzed; in that particular series, only two individuals revealed isolated adrenal medullary hyperplasia.

Clonality and Proliferation Features in Adrenal Medullary Hyperplasias

As mentioned above, there is no general agreement on the morphologic criteria to distinguish neoplastic (incipient pheochromocytomas) from nonneoplastic proliferations (hyperplasias) in the adrenal medulla [15]. On the other hand, most of our knowledge on adrenal medullary hyperplasias comes from a restrictive group of pathologic conditions, such as experimental pathology (in vitro and in vivo studies) and, to a lesser degree, from human pathology (in the case of inherited cancer syndromes). Those factors make it more difficult to obtain reliable and clinically relevant conclusions from the molecular analyses, useful for general application. Although no established conclusions are available at the molecular level, the proliferative response of the adrenal medulla is better known, at least under specific and controlled conditions [49–52].

Current evidences suggests that chromaffin cell proliferation in adult rats is regulated by a combination of hormonal and neurogenic signals [49,50]. The adrenal medulla is innervated by several nerve fibers, which stimulate the secretion of catecholamines. Therefore, after chronic administration of a wide variety of catecholamine-depleting pharmacologic agents, such as the antihypertensive agent reserpine, the releasing of negative feedback

controls increases neurogenic stimulation on chromaffin cells [50]. The reflexively increased neurogenic stimulation of chromaffin cells is intended to meet the physiological needs of catecholamine synthesis, and would result in adrenal medullary hyperplasia and neoplasia. Catecholamine depletion would be compensated through mechanisms that normally adjust cell number, increasing the cell turnover rate and ending in chromaffin cell outgrowths [50,52]. Many strains of rats develop similar changes spontaneously in the course of aging. These models have revealed, using different methods (mitotic figure counting and incorporation of 5-bromo-2'-deoxyuridine into replicating nuclei), a significant increase in the proliferation indices after reserpine stimulation, which can be partially prevented by adrenal denervation [49,52]. Denervation also causes a significant decrease in the proliferation index in nonstimulated animals. This hyperproliferative stage is postulated as a prelude to neoplastic transformation. The chronic persistence of these signals and superimposed abnormalities would lead to the selection of specific cell clones, which becoming dominant, explain the development and progression of neoplasms [50].

Conclusions

The currently available evidence on tumorigenesis and clonality in the adrenal medulla can be summarized as follows:

1. Multistep tumorigenesis defines the evolution of pheochromocytomas, as evidenced by the presence of several genetic alterations.
2. Both the significant association of non-random genetic alterations (specially 1p and 22q interstitial deletions) and the topographic accumulation of genetic deletions at the peripheral tumor compartment support a convergent clone selection for these neoplasms.
3. Although many genetic loci show nonrandom abnormalities, the most frequently involved genes locate on chromosome 1p, regardless of genetic tumor background (sporadic or inherited predisposition).
4. Most pheochromocytomas should begin as monoclonal proliferations that do not always correlate with histopathologic features, particularly in inherited tumor syndromes.
5. Early histopathologic stages, described as adrenal medullary hyperplasias, are defined by hyperproliferative features in animal models and monoclonal patterns in the adrenal nodules from patients with MEN-2a.

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