COMMENTARY

The **INK4a/ARF** locus in murine tumorigenesis

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**The **INK4a/ARF** locus is regarded as one of the most important anti-tumoral defenses that mammalian organisms possess.** The characterization of its two gene products, p16** \( ^{\text{INK4a}} \)** and p19** \( ^{\text{ARF}} \)**, has provided a great insight on the functioning of the tumor suppressors Rb and p53, respectively. Present evidence indicates that the **INK4a/ARF** locus is transcriptionally activated by oncogenic stresses, resulting in cell-cycle arrest or apoptosis. Here, I review the evidence accumulated on the involvement of the **INK4a/ARF** locus in murine tumorigenesis. Also, I summarize the phenotype of the different transgenic mouse models based on the inactivation of the **INK4a/ARF** locus.

**Introduction**

The p16** \( ^{\text{INK4a}} \)** protein was initially cloned as a protein associated with the cyclin D-dependent kinase CDK4, and it was shown to be an efficient inhibitor of the CDK4 kinase activity (1). Several proteins closely related to p16** \( ^{\text{INK4a}} \)** have subsequently been identified that together form the INK4 family: p16** \( ^{\text{INK4a}} \)**, p15** \( ^{\text{INK4b}} \)**, p18** \( ^{\text{INK4c}} \)** and p19** \( ^{\text{ARF}} \)** (2). The four INK4 proteins have similar biochemical activities, all of them binding and inhibiting CDK4 and its related kinase CDK6. The relevant feature that characterizes each INK4 protein appears to be its specific transcriptional regulation. In particular, the expression of p16** \( ^{\text{INK4a}} \)** is activated by oncogenic stresses (see below), while the expression of p15** \( ^{\text{INK4b}} \)** is upregulated by the negative growth factor transforming growth factor \( \beta \) (3).

One of the most surprising discoveries of recent years has been the realization that the **INK4a** locus contains an overlapping gene named **ARF** and encoding p19** \( ^{\text{ARF}} \)** (also called p14** \( ^{\text{ARF}} \)** when referring specifically to the human version) (4–7). The **INK4a** and **ARF** genes have their own separate promoters, each of which produces a different transcript: the **INK4a** transcript is formed by exons 1\( ^{\alpha} \), 2 and 3; and the **ARF** transcript is formed by exons 1\( ^{\beta} \), 2 and 3 (Figure 1). Although exons 2 and 3 are common to **INK4a** and **ARF** transcripts, they are read in different frames and, consequently, the corresponding proteins do not share amino acid sequence homology. The p19** \( ^{\text{ARF}} \)** protein has turned out to be a negative regulator of the p53-stabilizing oncogene MDM2 (8). The **INK4a/ARF** locus as a sensor of oncogenic stress

The normal levels of expression of p16** \( ^{\text{INK4a}} \)** and p19** \( ^{\text{ARF}} \)** are extremely low in most tissues. In mice, p16** \( ^{\text{INK4a}} \)** and p19** \( ^{\text{ARF}} \)**
expression is only detected in some adult tissues by RT–PCR (17,18). It appears, therefore, that these two tumor suppressors are not continuously restraining proliferation under normal conditions, but rather ‘awaiting’ to be activated in response to the appropriate signals. These signals have begun to be identified as oncogenic stresses (Figure 2). In particular, the presence of oncogenic Ras in a normal cell can activate the expression of both p16INK4a and p19ARF resulting in cell-cycle arrest (19–23). Also, other oncogenes, such as Myc, E2F1 and the adenosiral oncprotein E1a, activate the expression of p19ARF (but not of p16INK4a) (24–26). When expressed in normal cells, Myc, E2F1 and E1a, elicit a strong apoptotic response mediated primarily through activation of p53. It is now well established that the activation of p53 by Myc and E1a is mediated by the upregulation of p19ARF (24,25). In summary, the INK4a/ARF locus is activated in response to oncogenic stresses, thus preventing the propagation of cells carrying activated oncogenes (8,27).

Inactivation of the INK4a/ARF locus in mouse carcinogenesis

The inactivation of the INK4a/ARF locus has been thoroughly studied in human tumors. Essentially three modes of inactivation have been reported: (i) Homozygous deletion. This is a common mechanism of inactivation which generally involves the entire INK4a/ARF locus and very often the neighbor INK4b gene. It affects up to 14% of all human tumors analyzed (data obtained from ref. 2). (ii) Intragenic mutation. This type of inactivation mechanism is rather infrequent in the INK4a/ARF locus. Most point mutations occur in the second exon common for p16INK4a and p19ARF, usually affecting the amino acid sequence of both proteins. Nevertheless, a fraction of mutations occur in exon 1α, thus affecting exclusively the p16INK4a coding region. No mutations have been reported yet in exon 1β. All together, point mutations in the INK4a/ARF coding regions have been detected in 5% of all human tumors analyzed (data obtained from ref. 2). (iii) Promoter silencing by methylation. A significant fraction of human tumors show aberrant methylation of the INK4a promoter which results in a complete inactivation of its transcriptional activity. Silencing of the INK4a promoter by methylation has been found in 19% of all human tumors analyzed (data obtained from ref. 2). Aberrant methylation of the ARF promoter has been recently reported in human colorectal cancers independently of INK4a promoter methylation (28).

The status of the INK4a/ARF locus in murine tumors has been analyzed in a number of studies (summarized in Table I). Several interesting points can be made from the available data. In first place, the incidence of alterations in the murine INK4a/ARF locus appears rather low in primary solid tumors compared to the high incidence of alterations found in tumor cell lines. A similar situation has been encountered studying human tumors and derived cell lines. Two possible scenarios can be invoked to explain this situation. One possibility is that alterations in the INK4a/ARF locus are as frequent in primary tumors as in tumor cell lines, but their detection in primary tumors is often obscured by the presence of non-tumoral cells and/or by the different clonal populations that may form a tumor. In this regard, immunohistochemical analysis of p16INK4a in mouse lung tumors has shown the presence of focal areas within the tumor that lacked p16INK4a staining (29). Those tumor cells that have inactivated the INK4a/ARF locus may have a proliferative advantage with respect to the other tumoral cells and after in vitro expansion they may eventually dominate the culture. According to an alternative scenario, the inactivation of the INK4a/ARF locus is not present at all within the primary tumor, but occurs post-explantation during in vitro culture. However, a number of observations contradict this scenario and indicate that the establishment of tumor cell lines in vitro does not entail the inactivation of INK4a/ARF. For example, murine lung cancer cell lines of non-metastatic origin have a lower incidence of INK4a/ARF homozygous deletions compared with metastatic ones (30). Also, skin carcinoma cell lines of a differentiated phenotype have a very low (or zero) incidence of deletions, whereas similar cell lines of poorly differentiated phenotype show a high incidence of INK4a/ARF deletions (31; Table I). It appears that inactivation of the INK4a/ARF locus is generally a late event during tumorigenesis.

It is interesting to mention that a number of studies have found a significant proportion of INK4a/ARF intragenic mutations in lung and liver tumors induced with 3-methylcholanthrene (7 and 19%, respectively; Table I) (32–34), but not in similar tumors induced with other carcinogens (29,30,35). This is a remarkable observation considering the low incidence of intragenic mutations in the INK4a/ARF locus in human tumors.

A separate discussion must be made of murine lymphomas and leukemias. Regarding T-cell lymphomas, it appears that the loss of p15INK4b expression by promoter methylation is more relevant than the inactivation of the INK4a/ARF locus. This is particularly prominent in T-cell lymphomas induced by γ or neutron radiation (88 and 42% of INK4b promoter methylation, respectively) (36). These observations are in line with similar findings highlighting the pre-eminence of INK4b inactivation in specific human hematological malignancies (37–39). In any case, one study has analyzed the status of p16INK4a, p15INK4b, CDK4, cyclin D1 and Rb in the same set of lymphomas, reaching the conclusion that 75% of the radiation-induced T-cell lymphomas have alterations in one or several components of this pathway (40). Regarding B-cell lymphomas developed by transgenic mice expressing the Myc oncogene, there is strong evidence indicating that p19ARF is
the continued presence of an oncogenic stress, such as tumors it has been possible to map susceptibility loci with a high resolution in the mouse genome. For some particular types of tumors it has been possible to map susceptibility loci with a high resolution in the mouse genome. One of the susceptibility

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Frequency of INK4a/ARF alterations (%)</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma/leukemia</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T-cell (radiation)</td>
<td>0–1</td>
<td>Homozygous deletion</td>
<td></td>
</tr>
<tr>
<td>T-cell (chemical carcinogen)</td>
<td>6–25</td>
<td>Intragenic mutation</td>
<td></td>
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<tr>
<td>B-cell (MoMLV&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>0</td>
<td>Promoter methylation&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>B-cell (Eμ-myc&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>20–24</td>
<td></td>
<td></td>
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<tr>
<td>Liver cancer</td>
<td>0–6</td>
<td>0–7&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>n.d.</td>
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<td>Intraspecific mutations in exons 1α and 2β</td>
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<tr>
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<td></td>
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<tr>
<td>Derived cell lines</td>
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<tr>
<td>Skin cancer</td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>Derived cell lines</td>
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<sup>a</sup>ARF promoter methylation has not been examined in any of the studies listed, except in (62).
<sup>b</sup>Induced by infection with Moloney-Murine Leukemia Virus.
<sup>c</sup>Lymphomas/kemias produced in transgenic mice expressing Myc in B-cells.
<sup>d</sup>From spontaneous and chemically induced tumors.
<sup>e</sup>Intragenic mutations were found in tumors induced with 3-methylcholanthrene (32–34), but not in tumors induced with 4-(methyltritosamino)-1-(3-pyridyl)-1-butanone (29,30,35).
<sup>f</sup>Two tumors were analyzed and both had a homozygous deletion in agreement with the corresponding cell lines derived from them.
<sup>g</sup>Melanomas were induced in mice containing a transgene that expresses oncogenic Ras under the tyrosinase promoter (melanocyte specific) and treated with 7,12-dimethylbenz[a]anthracene.

### Table II. INK4a/ARF alleles and cancer susceptibility in mice

Many observations indicate that cancer susceptibility is under complex multigenic influences. For some particular types of tumors it has been possible to map susceptibility loci with a high resolution in the mouse genome. One of the susceptibility
loci for chemically induced plasmacytomas (a B-lymphocyte malignancy) has been proposed to correspond to the INK4a/ARF locus (47). Similarly, a susceptibility locus for chemically induced lung tumors has also been mapped to the INK4a/ARF locus (48). Interestingly, sequencing of the INK4a/ARF coding regions of ‘susceptible’ and ‘resistant’ mouse strains has shown that a variant allele is specifically present in the ‘susceptible’ strains (such as Balb/c). The variant allele contains two missense codons with respect to the canonical mouse sequence: one in exon 1α and the other in exon 2. Biochemical analysis of the corresponding p16INK4a and p19ARF ‘variant’ proteins has shown that the ‘variant’ p16INK4a protein is indeed impaired in its CDK4–6/cyclD inhibitory function (47,49). No defects were found in the ‘variant’ p19ARF protein. These observations indicate that the INK4a/ARF locus is a tumor susceptibility locus in mice.

Mouse tumor models based on targeted inactivation of the INK4a/ARF locus

Engineered alterations of the INK4a/ARF locus have served to test the function of p16INK4a and p19ARF in tumor suppression, and to generate sophisticated tissue-specific tumor models (Table II). Two mutations have been introduced so far into the INK4a/ARF locus: elimination of exons 2 and 3 (INK4a/ARFΔex2,3) (50), and elimination of exon 1β (ARFΔ1β) (51). In the first case, deletion of exons 2 and 3 completely eliminates the function of p16INK4a. However, the situation is not that clear regarding the status of p19ARF because INK4a/ARFΔex2,3 cells express at low levels a transcript containing exon 1β and encoding an active p19ARF mutant protein (C.Pantoja, I.Palmero and M.Serrano, manuscript in preparation). This raises the possibility that INK4a/ARFΔex2,3 mice could be partially functional for p19ARF. In any case, both mutant mice, INK4a/ARFΔex2,3 and ARFΔ1β, have strikingly similar phenotypes (50–52; Table II), which suggest that p19ARF is more relevant than p16INK4a for tumor suppression in mice. Notwithstanding, this issue will not be unambiguously answered until the generation of new mutations that specifically inactivate p16INK4a without affecting p19ARF.

Several mouse tumor models have been generated by combination of the above-mentioned mutations with transgenic oncogenes expressed in specific cell types. Importantly, these mice reproduce the oncogenic cooperation previously demonstrated in cell culture systems. For example, INK4a/ARFΔex2,3 mice have a dramatically increased susceptibility to the oncogenic effects of Ras expressed in melanocytes (53,54), or to the EGF receptor expressed in glia precursors (55) (Table II). Also, heterozygous INK4a/ARFΔex2,3 or ARFΔ1β mice in combination with a Myc-encoding transgene expressed in B-lymphocytes develop acute lymphomas with a very short latency (41–43) (Table II).

It is interesting to mention that INK4a/ARF haplo-insufficiency may have strong effects on tumorigenesis (43,55). Remarkably, gliomas were produced equally efficiently in homozygous and heterozygous INK4a/ARFΔex2,3 mice expressing an activated EGF receptor in glia precursors (55). Since this is not a general effect in other tumor types, such as melanomas (53), it appears that different cell types have different sensitivities to changes in the genetic dose of INK4a/ARF.

Concluding remarks

The role of the INK4a/ARF locus in tumor suppression is now relatively well understood. The manipulation of the INK4a/ARF locus in the mouse has already yielded interesting and sophisticated tumor model systems. New manipulations of the INK4a/ARF locus will certainly provide a more clear picture, and will serve to generate additional tumor model systems.

Acknowledgements

The author would like to express his appreciation to David Beach (Cold Spring Harbor Laboratory) in whose laboratory the author made most of his contributions to this study. The laboratory of M.S. is supported by the Spanish Research Council, the Spanish Ministry of Education, the Regional Government of Madrid, the Human Frontier Science Program, and a core grant to the Department of Immunology and Oncology from the consortium between Pharmacia & Upjohn and the Spanish Research Council.

References


Received January 31, 2000; accepted February 1, 2000