Carcinogenesis is a multistage process driven by activation of oncogenes and inactivation of tumor suppressor genes. Differential expression of these critical genes and other genes controlled by them contributes to malignant transformation. The identification of these genes is essential for understanding the molecular mechanism of carcinogenesis. We have developed an in vitro murine epidermal cell transformation model (1) in which multiple stages have been derived from a clonal lineage. A normal cloned epidermal cell strain (291) sensitive to calcium-induced terminal differentiation (2) was treated with the chemical carcinogen 7,12-dimethylbenz[a]anthracene (DMBA*), and three independent initiated cell clones (291.03C, -05C, and -09C) were isolated based on altered response to extracellular calcium (3). Following the treatment of the resulting cell lines with retinoic acid and their transplantation to athymic mice, three tumor types were generated: a benign papilloma (291.09RA T [09RA T]), a well differentiated squamous cell carcinoma (05RA T), and a poorly differentiated squamous cell carcinoma which is invasive and metastatic (03RA T) (4). This epidermal cell model has a number of characteristics that are advantageous in the identification of alterations during carcinogenesis. Firstly, cells at different stages of carcinogenesis are available to dissect the order and consequences of genetic alterations. Secondly, cells to be compared can be maintained under identical growth conditions. Thirdly and more importantly, the normal, pre-tumorigenic and tumorigenic cells have a common genetic background. Therefore, this model was used to attempt to identify alterations that are specific to carcinogenesis.

Altered gene expression patterns of 291, 03C, 03RA T, and 09RA T cells were compared by RNA differential display (5). A feature of RNA differential display is its ability to identify both overexpressed genes and suppressed genes among multiple samples simultaneously. Therefore it lends itself to identifying activation of oncogenes and inactivation of tumor suppressor genes at different stages of tumorigenesis. Usually, the initiated and tumorigenic cells are maintained in medium containing 1.4 mM Ca^{2+} (high calcium medium), whereas 291 cells are cultured in medium containing 0.04 mM Ca^{2+} (low calcium medium) which maintains 291 cells in a proliferative state (1). To normalize the growth conditions, the initiated and tumorigenic cells were switched to medium identical to that of 291 cells two days before they reached 70% confluency. The mRNA expression patterns were analyzed by RNA differential display with 12 different combinations of primer sets made of anchored oligo (dT) primers and randomly designed arbitrary primers (Genhunter). Theoretically only 5% of the mRNA population in cells can be displayed by 12 primer combinations (5), yet this provides a window through which to view the dynamic process of genetic alterations during multistage carcinogenesis. A total of 17 potential differentially expressed genes were detected in initiated, malignant tumor, and the benign tumor cells as compared with the normal parental cells. Patterns of displayed bands between the normal cell and the initiated cell were almost identical, consistent with their clonal origin. Only one overexpressed gene was found, indicating good reproducibility of the technique between independent experiments and very few alterations during the initiation stage. However, most differentially displayed bands were observed between the normal cell and the malignant cell, with 15 out of 17 differentially displayed bands isolated being malignancy related. The dramatic accumulation of alterations during the progression from the initiation stage to malignancy suggests the malfunction of certain critical genes involved in DNA repair, transcriptional regulation, or cell cycle checkpoints. It has been demonstrated previously that alterations in

*Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; ARK, adhesion-related kinase; Gas6, growth arrest specific gene 6; PCR, polymerase chain reaction; TPA, tetradecanoylphorbol-13-acetate; SCC, squamous cell carcinoma.
Altered gene expression detected by RNA differential display. The murine epidermal cell lines indicated were cultured until 70% confluent. Cells were harvested and purified total RNAs were subjected to reverse transcription followed by PCR amplification using primer set AP7/T11G (Genhunter) in the presence of $^{35}$S-dATP. The PCR products were fractionated and displayed on a 5% polyacrylamide sequencing gel. Differential loss 7G1 and a differential gain (arrowhead) are shown.

RsaI restriction fingerprinting of differentially displayed 7G1 clones. The plasmids from positive clones of 7G1 were subjected to RsaI digestion and electrophoresis through a 1.0% agarose gel. Based on the digestion patterns, the plasmids were sorted into three groups, A, B and C, as indicated.

p53 RNA and protein levels occur at the malignant conversion stage (6). The p53 loss has been associated with genetic instability by *in vitro* experiments (7,8) and cytogenetic analysis of human cancer (9). The profile of alterations observed in this model is consistent with the association of genetic instability with p53 functional loss and malignancy.

Two differentially displayed fragments in the 03RAT cells, a loss and a gain, are shown in Figure 1. The gain was verified by northern analysis and identified as a novel gene of AAA-protein family (ATPase associated with diverse activities) (unpublished data). Fragment 7G1 was excised from the 291 band in the polyacrylamide gel and reamplified by PCR with the same primer set used in the original RNA differential display. The amplified DNA fragment was cloned directly into the pBluescript plasmid (Stratagene) treated with EcoRV/Taq polymerase. A problem often encountered in RNA differential display is that the DNA fragments isolated from the original sequencing gel are contaminated with other same-sized DNA fragments.

Northern analysis of 7G1 (ARK) expression in the cloned cell model of carcinogenesis. Ten µg of total RNA from each cell type shown were separated by 1.2% agarose formaldehyde gel electrophoresis. The blot was hybridized with random-primer $^{32}$P-labeled 7G1 isolated from RNA differential display and autoradiographed at -70°C for 2 days. For RNA loading control, the blot was rehybridized with 7S RNA (32) and autoradiographed at room temperature for 2 h.

Comparison of 7G1 nucleotide sequence with murine adhesion kinase (ARK) (Genbank accession # X59560). 7G1 DNA fragment was sequenced from both directions by the dideoxy method using M13 forward and reverse primers (USB). The 7G1 sequence was used to search Genbank database and compared with ARK by means of the GCG Bestfit program. 7G1 sequence is 99% homologous to the 3′ untranslated region of ARK.
fragments, thus false positive clones may be randomly selected (10). The true positive clones were selected by restriction fingerprinting as described (11). Statistically, the cutting frequency for a 4 bp-cutter restriction enzyme is one in 256 bp; therefore a 4 bp-cutter restriction enzyme could be applied in analyzing DNA fragments >400 bp. Based on the restriction pattern with the 4 bp-cutter restriction enzyme RsaI, plasmids containing inserts from the 7G1 cDNA fragment were grouped into three distinct forms, A, B and C (Figure 2). Since five out of nine 7G1 plasmids tested were of group A, plasmids of this group were selected for Northern analysis.

Northern analysis of blots containing total RNA of 291, 03C, 03RA T, 05RA T, and 09RA T with cloned 7G1 as probe revealed ~4.5 kb transcript that was barely detectable in malignant cell 03RA T (Figure 3). The sequence of 7G1 showed 99% homology to the 3’ untranslated region of murine receptor tyrosine kinase (Figure 4), an adhesion-related kinase (ARK) with the capability of promoting cell aggregation through homophilic binding (12). Its human counterpart, AXL or UFO (13,14), was identified as oncogenic by DNA-mediated transformation of NIH 3T3 cells with DNA from patients with chronic myeloid leukemia. AXL/ARK is expressed in hematopoietic progenitor cells and leukemic cells of myeloid and erythro-megakaryocytic lineages, but not in normal peripheral blood cells and lymphoid leukemias (15,16). Increased expression of AXL/ARK is observed in CML cell K562 during cell differentiation induced by TPA, but not in HL60 cell treated with DMSO. This cell type-dependent expression of AXL/ARK was also observed in nonhematopoietic tumors. Overexpression of AXL/ARK has been documented in human colon cancers (17). However suppressed expression of AXL/ARK was also found in certain other tumors including the undifferentiated teratocarcinoma F9 (18) and the poorly differentiated squamous cell carcinoma 03RA T in this study. AXL/ARK receptor has recently been found to be bound and activated by growth arrest specific gene 6 (Gas6) (19), a gene originally identified as being induced in response to growth arrest in NIH 3T3 mouse fibroblasts and IMR90 human fibroblasts (20). Nevertheless Gas6 showed moderate growth-promoting effects in some cells (21,22). These observations suggest that AXL/ARK may have dual functions in cellular proliferation and differentiation depending upon the cell type, the growth or differentiation state, and the presence of ligands. It has been postulated that AXL/ARK may heterodimerize with another receptor which determines which SH-2 containing molecules are to be recruited for differential downstream signaling (23). Therefore different biological responses may be mediated by the AXL/ARK receptor upon heterodimerization with different receptors on the cell surface. In the current study, AXL/ARK was expressed in normal epidermal cells having the properties of epidermal progenitors and sensitive to calcium-mediated terminal differentiation. Suppressed expression of AXL/ARK in tumor cells was associated with the undifferentiated phenotype in SCC 03RA T, whereas AXL/ARK expression was maintained in tumorigenic cells (moderately differentiated SCC 05RA T, and papilloma 09RA T) with differentiation potential. Studies in somatic cell hybrids among the cell lines in our model system showed that the tumorigenicity of 03RA T cells can be abolished by nontumorigenic cell 291, and can be suppressed to some extent by benign tumor cell 09RA T and moderately differentiated tumor cell 05RA T (24). Tumor suppressor phenotypes in these hybrids apparently involved gain of cellular differentiation activity as indicated by the presence of differentiation-specific keratin gene K1 in 03RA T×291 hybrids, and highly differentiated morphology in tumors generated from 03RA T×09RA T and 03RA T×05RA T (24), consistent with the results of others (25). The loss of AXL/ARK in 03RA T cells may contribute to malignant conversion by retaining cells at an earlier differentiation stage with greater proliferative potential. This speculation is being tested by examining the expression patterns of AXL/ARK during epidermal cell differentiation and the tumorigenicity of 03RA T cells transfected with ARK cDNA.

Compelling evidence from clinical and experimental models of carcinogenesis indicates that more than two genetic alterations involving activation of oncogenes and inactivation of tumor suppressor genes are required for malignant transformation (26,27). In the absence of Ha-ras mutation in this cloned keratinocyte model (4), identifying alternative genetic events which cooperate to confer the transformed and malignant phenotypes are of potential significance to understanding the genes driving human epithelial cell transformation, in which ras gene mutations are infrequent (28,29). The only functional alteration which has been associated with malignant transformation in the cloned epidermal cell model to date is altered expression of the p53 tumor suppressor gene (6). Although the loss of p53 is the most frequent genetic event in malignant transformation, p53 defects alone are not sufficient for initiation and tumor formation. In two-stage skin carcinogenesis experiments, a high frequency of carcinoma formation was observed in mice with p53 null mutations, whereas no tumors developed in the p53 null mice treated with TPA alone without DMBA initiation (30). Furthermore, no tumors developed in p53 null transgenic mice bearing epidermal-targeted v-Ha-ras treated with TPA only (31), indicating that additional genetic alterations are required. The current study has identified a loss of ARK at the malignant conversion stage of epidermal carcinogenesis. Future work will focus on whether ARK or other differentially displayed genes are functional in malignant conversion, the undifferentiated phenotype or genetic instability or secondary to these processes.

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