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Genes Differentially Expressed With Malignant Transformation and Metastatic Tumor Progression of Murine Squamous Cell Carcinoma

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Abstract Molecular changes occurring with tumor formation and metastasis need to be identified in order to define novel markers and targets for chemoprevention and therapy. Cell lines from a multistage model of murine squamous cell carcinoma were analyzed for differences in gene expression using mRNA differential display. mRNA was isolated from primary keratinocytes, an in vitro transformed keratinocyte line (Pam 212), and three metastatic cell lines derived from Pam 212 following tumor progression in vivo. cDNA was synthesized by reverse transcription and amplified by PCR using 72 primer combinations to screen and compare approximately 3,600 sequences. Five cDNAs with a differential expression pattern confirmed by Northern blot analysis were cloned and sequenced, revealing homology with known genes. The gene encoding tropomyosin α was preferentially expressed in primary keratinocytes; genes for tyrosine kinase Yes-associated protein (YAP65) and ribosomal protein L18a were preferentially expressed in transformed and metastatic tumor cell lines; and genes for the Gro-α family cytokine KC and antigen Sp17 exhibited increased expression in the three metastatic cell lines. The structure and function of the genes identified suggest that they may possibly be linked to cell shape and motility, signal transduction, protein synthesis, growth, granulocyte chemotaxis, and angiogenesis. This study demonstrates the ability of mRNA differential display to detect altered gene expression in this tumor progression model of murine squamous cell carcinoma, and the potential usefulness of this approach for identification of candidate genes as chemoprevention markers and targets. J. Cell. Biochem. Suppls. 28/29:90–100. © 1998 Wiley-Liss, Inc.†

Key words: squamous cell carcinoma; differential display; tropomyosin α; cytokine KC; ribosomal protein L18a; antigen Sp17

Malignant transformation and tumor progression of squamous cell carcinoma (SCC) is characterized by a series of changes in pathological and clinical behavior for which the molecular mechanisms remain to be defined. During the course of disease, increased proliferation, altered recognition, local invasion, and a subsequent rapid progression in growth and metastasis, is often observed. Such progressive changes in behavior are thought to result from an accumulation of genetic alterations [1–3]. Altered expression of gene products in a number of pathways may contribute to the changes in pathologic behavior, including pathways involved in cell recognition and adhesion, signal transduction, cell growth and death, angiogenesis, and host immunity. The identification of these molecular changes is important in understanding the mechanisms involved in tumor behavior, and in defining novel markers and targets for cancer diagnosis, chemoprevention, and therapy.

Progress in studying the molecular pathogenesis of SCC has been aided by the development of well-defined models, especially models that reflect the neoplasm at different stages of tumor development and progression [4–6]. A syngeneic animal model of SCC could facilitate identification of genetic changes necessary for escape from both cellular and host control mechanisms on a defined genetic background. We have developed a multistage experimental model of SCC to help distinguish pathologic behavior associated with primary events in ma-

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lignant transformation, and those associated with adaptation in the host that contribute to rapid tumor progression and metastasis [7]. The model was established from the murine cell line Pam 212, which was derived by spontaneous transformation of neonatal BALB/c keratinocytes in vitro in the absence of host factors [8]. In vivo, Pam 212 produces a locally invasive squamous cell carcinoma, which rarely gives rise to metastases [9].

In the present study, we compared the expression of genes in nontransformed BALB/c keratinocytes, transformed Pam 212, and cell lines isolated from rare metastases of Pam 212 by RT-PCR differential display (DD). The majority of cDNAs derived were co-expressed by all of the cell lines, providing evidence for derivation of the cell lines from the same epithelial lineage and strain of origin. Rare cDNAs that were differentially expressed between the nontransformed and transformed cell lines, and between the transformed and metastatic variant cell lines, were also detected following Northern blot analysis. Sequences of five cDNA fragments revealed a match with known genes; four of the five isolated genes possess structural or functional properties associated with cell motility, signal transduction, protein synthesis, growth, granulocyte chemotaxis, and angiogenesis, making them appropriate candidates for pathways involved in tumor suppression or oncogenesis.

MATERIALS AND METHODS

Nontransformed, transformed, and metastatic BALB/c cell lines had the following origin and characteristics: Nontransformed keratinocytes were cultured from the skin of male BALB/c neonates, as described elsewhere [8], while the Pam 212 cell line, a spontaneously transformed keratinocyte line, was derived by selection of BALB/c keratinocytes in media containing a high calcium concentration in vitro [8]. The Pam 212 line forms a locally invasive squamous cell carcinoma, but rarely metastasizes when recipients are inoculated with a tumorigenic dose of 5 x 10^6 cells [9]. The isolation and characterization of metastatic Pam 212 lines have recently been described elsewhere [7]. The Pam 212 and reisolate cell lines were maintained in EMEM plus 10% fetal calf serum and antibiotics in monolayer culture. All cell lines were tested and found to be free of mycoplasma contamination.

RNA ISOLATION AND DIFFERENTIAL DISPLAY OF mRNA

Differential display was performed based upon the method of Liang and Pardee [10]. Total RNA was isolated from cultured primary keratinocytes, Pam 212, Pam LY-1, Pam LY-2, and Pam LU-1 tumor cells when 80–90% confluent on 75 or 150 cm^2 tissue culture flasks, using Trizol reagent according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). DNase I-treated RNA (0.2 µg) from each cell line was used in differential display with the RNAimage kit (GenHunter Co., Brookline, MA) following the manufacturer's instructions. Briefly, reverse transcription of mRNA was performed with the anchor primers H-T_{11}G, H-T_{11}A, or H-T_{11}C in a 20 µl reaction containing 25 mM Tris-Cl, pH 8.3, 37.6 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, 20 µM dNTPs and MMLV reverse transcriptase. The resulting cDNAs were amplified by PCR with each anchor primer in combination with H-AP1 to H-AP8, and H-AP17 to H-AP32 in the presence of 0.25 µl of [α-^{33}P]dATP (2,000 Ci/mmol, Redivue, Amersham, Chicago, IL). Samples were resolved on a 6% urea polyacrylamide gel followed by autoradiography. Bands identified and recovered from the sequencing gel were reamplified with the original primer set and run on 1.5% agarose gel.

DNAs were recovered from the gel by excision, and purified using the Geneclean II kit (BIO 101, Inc., Vista, CA). The pattern of specificity and size of mRNAs that hybridized with the cDNAs was confirmed by Northern analysis prior to cloning, to eliminate false positives and select candidates, and repeated after cloning, to confirm isolation of the expected fragment. To optimize detection sensitivity for hybridization with the uncloned PCR products, we used 1.5 µg of purified mRNA isolated from cultured neonatal BALB/c keratinocytes, Pam 212 cells, and reisolates LY-1, LY-2, and LU-1, using the FastTrack 2.0 kit (Invitrogen, San Diego, CA). To verify the hybridization pattern of products following cloning, 20 µg of total RNA from the same cell lines, isolated as described for DD above, was used. The RNA from each cell line was resolved by electrophoresis on a 1.2% formaldehyde-agarose gel, and Northern blotting was performed using standard methods [11]. After stripping, the blots were used for four rounds of hybridization. Each blot was then probed with labeled GAPDH cDNA (Clontech

Laboratory, Inc., Palo Alto, CA) to confirm equal loading and the integrity of the RNA run on the gels.

The gel-purified DNA fragments from DD were cloned into the plasmid vector pCR™ 2.1 using the TA cloning kit (Invitrogen, San Diego, CA), according to the manufacturer's instructions. Five to ten white colonies grown on LB plates with X-Gal were routinely picked for each candidate cDNA, heated in 50 µl of water at 100°C for 2 min, and, after spinning briefly, 2 µl of supernatant was used for PCR with M13 reverse and T7 primers. PCR products were resolved on a 1.5% agarose gel to identify colonies containing plasmids with inserts. Cultures of bacteria were inoculated with positive colonies and plasmid DNA was isolated from overnight culture using the QIAwell 8 Plus Plasmid Kit (QIAGEN Inc., Chatsworth, CA). Sequencing of miniprep DNA was performed with either M13 reverse primer or T7 primer using the Dye Terminator ABI PRISM Kit (Perkin Elmer, Foster City, CA) and analyzed on an ABI 373 DNA sequencer (Applied Biosystems Inc., Foster City, CA). Alignment of insert sequences obtained was made using the MacDNASIS program (Hitachi Software Engineering America, Ltd., San Bruno, CA), and the BLAST program was used to search for homologous sequences in Genbank.

RESULTS

To distinguish phenotypic differences due to cellular transformation from those due to host adaptation and metastatic tumor progression, we recently developed a model based on the Pam 212 cell line transformed in vitro, and on metastases of Pam 212 obtained following metastatic tumor progression in vivo [7]. Reisolated lines obtained from lymph node metastases were designated as Pam LY; those obtained from lung were designated as Pam LU. Keratinocyte lineage and cellular differentiation of the Pam 212 and reisolate cell lines were confirmed by comparing the expression of cytokeratins K6 and K14 with integrin β4 subunit. As summarized in Table I, the Pam 212 cell line and all reisolates expressed cytokeratin K6 and K14 mRNA and protein, indicating that all cell lines expressed markers of a squamous epithelial origin. All cell lines exhibited uniform immunostaining with β4 integrin antibodies, expressed by epithelia but not fibroblasts [12–14]. Immunostaining of cytokeratin and integrin markers by the cell population within each of the cell lines showed no contamination with individual cells or colonies of cells which did not express the markers (data not shown). However, a general decrease in cytokeratin staining and mRNA expression with tumor progression in order of nontransformed > transformed > metastatic cell lines was noted, consistent with variation in cytokeratin differentiation of the cell lines.

In vivo, the reisolated cell lines exhibited a more aggressive tumorigenic phenotype than the parental tumor line Pam 212. As summarized in Table I, tumors from Pam reisolates grew more rapidly than the parental Pam 212 cells, and exhibited a five- to tenfold difference in tumor size within 1 month, when recipients of the reisolate lines began to succumb to progressive disease. The recipients of the reisolated cell lines also had a shortened median survival when compared with recipients receiving an equivalent dose of the Pam 212 parental cell line. Furthermore, the recipients of the reisolated tumor lines exhibited a higher incidence of metastatic tumor formation than recipi-

TABLE I. Characterization of Pam 212 and Reisolate Cell Lines Used for Differential Display*

Cell lines	Cytokeratin[a] K6	Cytokeratin[a] K14	Integrin β4 subunit[b]	Tumor size [31 days (cm^2)]	Average survival (days)	Metastasis (incidence)[c]
Pam 212	+++	+++	+++	0.03 ± 0.02	>92	0/15
Pam LY-1	+	++	+++	0.97 ± 0.11	46.2	3/5
Pam LY-2	++	++	+++	0.75 ± 0.12	48.6	2/5[d]
Pam LU-1	++	++	+++	0.56 ± 0.14	40.4	3/5

*The isolation and characterization of metastatic Pam 212 lines has recently been described by Chen et al. [(7)].

[a]Keratin K6 and K14 expression was determined by Northern blot analysis and immunoperoxidase staining and scored as low (+), intermediate (++), and high (+++) by comparing the intensity of signal by immunostaining and by Northern Blot.

[b]β4 integrin subunit immunostaining was determined by flow cytofluorometry.

[c]Incidence of lymph node and/or lung metastases in recipients of Pam 212 and reisolate cell lines. The presence of metastasis was determined at the time of euthanasia for morbidity in recipients of reisolates (days 18–62) or at the time of termination of the experiment for Pam 212 (day 92).

[d]Incidence of metastasis was not evaluable in 2/5 recipients due to premature death.

ents of the parental Pam 212 cell line at an equivalent cell dose (Table I). The site of metastases in recipients included both lymph node and lung, without apparent respect to whether the reisolate was derived from LY or LU sites (data not shown). Thus, the reisolate cell lines obtained following malignant tumor progression in the syngeneic host appeared to exhibit a more aggressive phenotype than the parental tumor when implanted in vivo.

COMPARISON OF mRNA EXPRESSION IN Pam 212 AND REISOLATES BY DIFFERENTIAL DISPLAY

To determine if differences in mRNA expression correlated with transformation and tumor progression in the model system, we used the RT-PCR differential display screening strategy developed by Liang and Pardee [10], as described in Materials and Methods. Among approximately 3,600 bands generated by using 72 primer pairs in DD, more than 95% were consistently displayed in all cell types. Twenty-five bands reproducibly showed differential expression in these experiments. Figure 1 shows representative displays of five bands that were subjected to further studies. Band 20 and band 75 were expressed more intensely in tumor lines than in nontransformed keratinocytes. Band 24 and the doublet band 34 were not detected in normal keratinocytes or in the parental Pam 212 cell line, but were amplified in all three of the reisolates. Each of the bands comprising the doublet was recovered separately and analyzed (see below). Band C1, a doublet, displayed increased intensity in normal BALB/c keratinocytes, but was not detected among any of the tumor cell lines. Thus, the differential display results demonstrated apparent differences in gene expression associated with the stepwise changes observed in the phenotypic behavior of cell lines following transformation in vitro, and metastatic tumor progression in vivo.

NORTHERN BLOT ANALYSIS OF DNA FRAGMENTS IDENTIFIED BY DD

To verify whether candidate cDNAs generated by DD would detect changes in gene expression in cell lines of differing neoplastic potential, Northern blot analysis was carried out using different isolates of mRNA obtained from

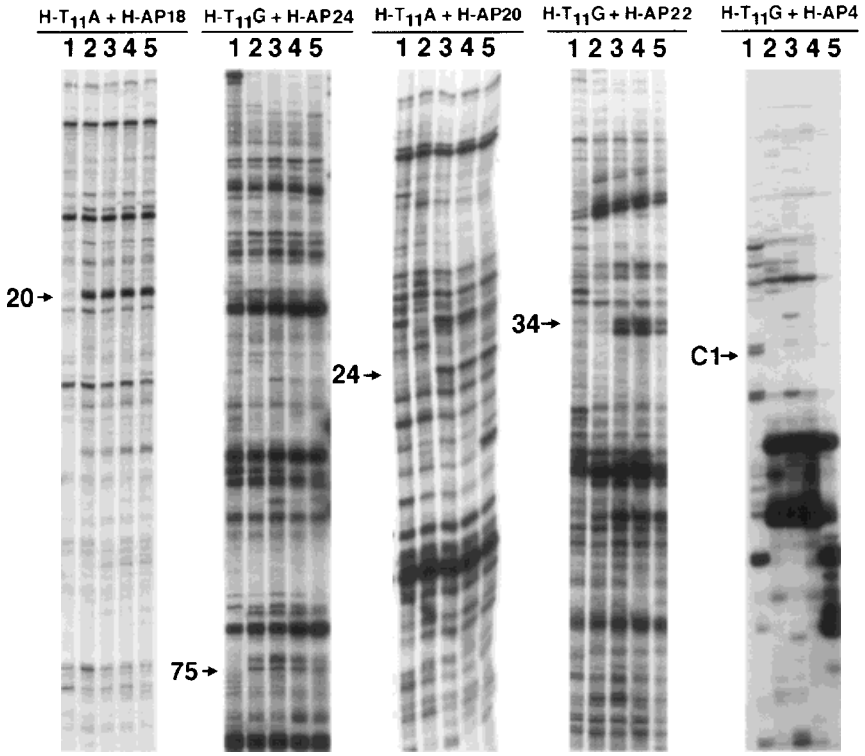

Fig. 1. mRNA differential display comparison of Pam 212 and metastatic reisolate lines. Differential display was performed as described in Materials and Methods using total RNA of BALB/c primary keratinocytes (1), Pam 212 (2), Pam LY-1 (3), Pam LY-2 (4), and Pam LU-1 (5), and resolved on a 6% sequencing gel. Primers used in DD are listed on top of each panel. Candidate bands are marked by arrows and were designated in numerical order of detection.

the same cell lines used in DD. The five DNA fragments recovered from DD gel were reamplified, gel purified, and labeled as probes for Northern blotting. As shown in Figure 2, probe 20 detected mRNAs with a strong 4.0 kb signal and a weak 2.5 kb signal, present in all tumor lines with equal intensity, a pattern consistent with DD. Signals were not detected in RNA from primary cultured keratinocytes following prolonged exposure of the same blot (data not shown). Probe 75 detected a single RNA band below 1 kb. This single species of RNA could be detected in all of the cell lines, but showed a fivefold increase in signal intensity in tumor lines when the activity in the blot was quantified using an InstantImager (data not shown). Probe 24 detected a strong RNA signal around 1 kb in size in reisolated cell lines. Upon prolonged exposure, an RNA signal of the same size could be detected in the Pam 212 lane, but with much reduced intensity. A faint band around 2 kb also appeared in this Northern blot but was not reproduced in a later experiment (data not shown). The two probes from doublet 34 recovered separately from DD detected a single RNA species of the same size below 1 kb, which was present only in the reisolates. Probe C1 detected an RNA band around 1.3 kb in primary cultured keratinocytes. The intensity of the signal was reduced in tumor lines by threefold or larger (data not shown). To exclude the possibility that the differentially expressed cDNAs obtained from the tumor lines in this study were amplified from a subpopulation of cells of non-keratinocyte origin acquired during selection in vivo, we also determined the hybridization of the cDNAs to RNA obtained from cloned cell lines derived from Pam 212 and Pam LY-1. The cDNA for bands 24 and 34 exhibited a similar pattern of expression in the panel of cell lines and subclones, and we verified the keratinocyte origin of the cloned lines by demonstrating that the clones express cytokeratin K6 and K14 mRNA and proteins [15] (data not shown). Thus, Northern blot analysis confirmed that the candidate cDNAs exhibit the same pattern of expression observed in DD, and these candidates were selected for cloning and sequence analysis.

CLONING AND SEQUENCING OF DNAS

Since it has been reported previously that a single band in DD may contain more than one DNA population, we cloned DNAs recovered from DD and picked at least five individual colonies for sequencing, as described in Materials and Methods. We confirmed that the cloned cDNAs and original PCR-amplified cDNAs identified the same mRNA species by repeat Northern analyses with total RNA; reproducible results were obtained (data not shown). Sequence analysis of the cloned cDNAs from the colonies we analyzed showed that the sequence of the clones from each DD product shared greater than

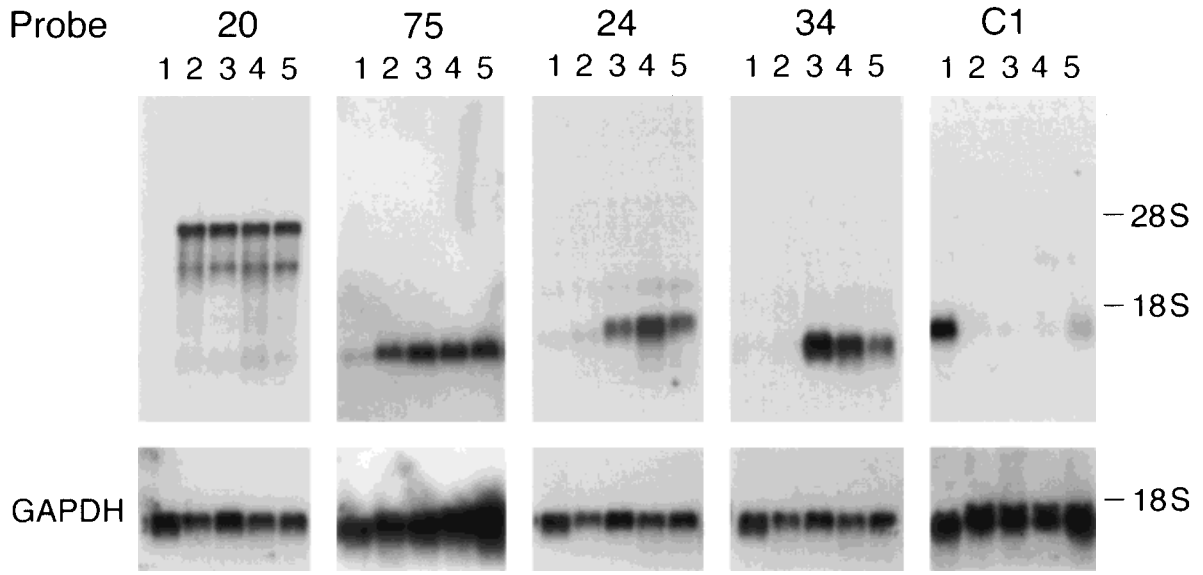

Fig. 2. Northern blot analysis of candidate cDNAs from DD. 1.5 µg of mRNA from cell lines shown in Figure 1 was analyzed by Northern blot using ^{32}P-labeled cDNA probe derived from reamplified cDNAs as designated. After a high stringency wash, each blot was exposed to X-ray film with an intensification screen at -80^{0}C from 1 h to 10 days. The same blots were stripped and reprobed for GAPDH to verify RNA loading.

95% homology following sequence alignment, suggesting that each DD fragment arose from a single species. Consensus sequences were deduced and used for a search of GenBank for homologous genes using the BLAST program. As shown in Figure 3, the sequence of fragment 20 was identical to that of the murine Yes-associated protein (YAP65) cDNA between nt 2144 and nt 2496 [16], except for incorporation of two extra C adjacent to the primer. Band 75 shared 93% homology with the 3′ portion of rat ribosomal protein L18a [17]. Band 24 shared 98% homology with the 3′ portion of the cDNA which encodes murine cytokine KC [18], a C-X-C cytokine with high homology to human growth-related gene *gro* and IL-8. Band 34 matched a large segment of the 3′ coding region and the entire 3′ UTR of the murine Sp17 antigen cDNA [19]. Band C1 was identical to the 3′ portion of murine tropomyosin-α cDNA between nt 867 and nt 1159 [20].

DISCUSSION

Using mRNA differential display in a murine model of SCC, we have identified five cDNAs which are differentially expressed in these nonmalignant, malignant, and highly malignant metastatic tumor lines (Fig. 1). The differences in expression of the amplified sequences were confirmed by demonstration of similar differences in the abundance of steady-state mRNA among the cell lines by Northern analysis (Fig. 2). The expression pattern of mRNA detected by the five DNA fragments from DD was consistent with stepwise changes in the phenotype associated with transformation and tumor progression (Table II). Thus, murine tropomyosin α mRNA 1.3 kb isoform (band C1) was detected in nontransformed keratinocytes but was down-regulated in the tumor lines; expression of mRNA for YAP65 (band 20) and ribosomal protein L18a (band 75) was elevated following cellular transformation and retained in all tumor lines; and mRNAs for KC (band 24) and Sp17 (band 34) were preferentially expressed following metastatic tumor progression in all three of the reisolate lines studied. These results demonstrated the potential of using the Pam 212 model and DD to detect changes in gene expression associated with transformation and tumor progression of SCC.

The sequences of the five cDNAs, which exhibit the stepwise changes in expression found in this study, match the sequences of five different genes that have been previously identified (Fig. 3 and Table II). The candidates identified include genes that may potentially be involved in determining cell shape and motility, signal transduction, protein synthesis, growth, granulocyte chemotaxis, and angiogenesis. The link between knowledge concerning function of these genes and tumor development and progression has yet to be made, but the nature of the putative function of some of the these genes based upon structural and functional data makes them interesting candidates for further study as markers and targets for chemoprevention or therapy.

Tropomyosin α (TM) is a member of a family of proteins with actin-binding activity expressed in muscle and nonmuscle cells that are important in cellular cytoskeletal organization and migration [21,22]. The exact function of TM family proteins in nonmuscle cells and neoplasia is still unclear. A loss or decrease in expression of certain TM isoforms has been noted in transformed cells and tumors [23–26] leading to the hypothesis that TM may function as a tumor suppressor gene. Decreased expression of TM isoforms has been reported in mammary carcinoma cell lines by Northern Blot analysis [26] and decreased TM β isoform expression in prostate cancer was recently demonstrated using DD [27]. Direct evidence for a tumor suppressor function by the TM β isoform has been reported by Braverman et al., 1996, who showed that expression of TM β was sufficient to suppress growth of *v-Ki-ras*-transformed NIH3T3 cells in soft agar [28]. Interestingly, expression of the TM α coding sequence had no effect on growth of transformed NIH3T3 cells [28], but overexpression of 3' UTR of TM α has been correlated with the phenotype reversion of other transformed cells [29]. The potential anti-tumor activity of TM has not been studied in SCC. Normal keratinocytes expressed a 1.3 kb TM α isoform, but expression decreased in the Pam 212 tumor model, suggesting that down-regulation of this isoform may play a role during keratinocyte transformation. If proven, the effect of TM isoforms may be cell-type dependent. It will be interesting to determine if expression of TM in nonexpressing transformed Pam 212 cells will affect the phenotype, including organization of integrin and cadherin cell adhesion molecules, whose interaction with cytoskeletal proteins appear to be abnormal following transformation.

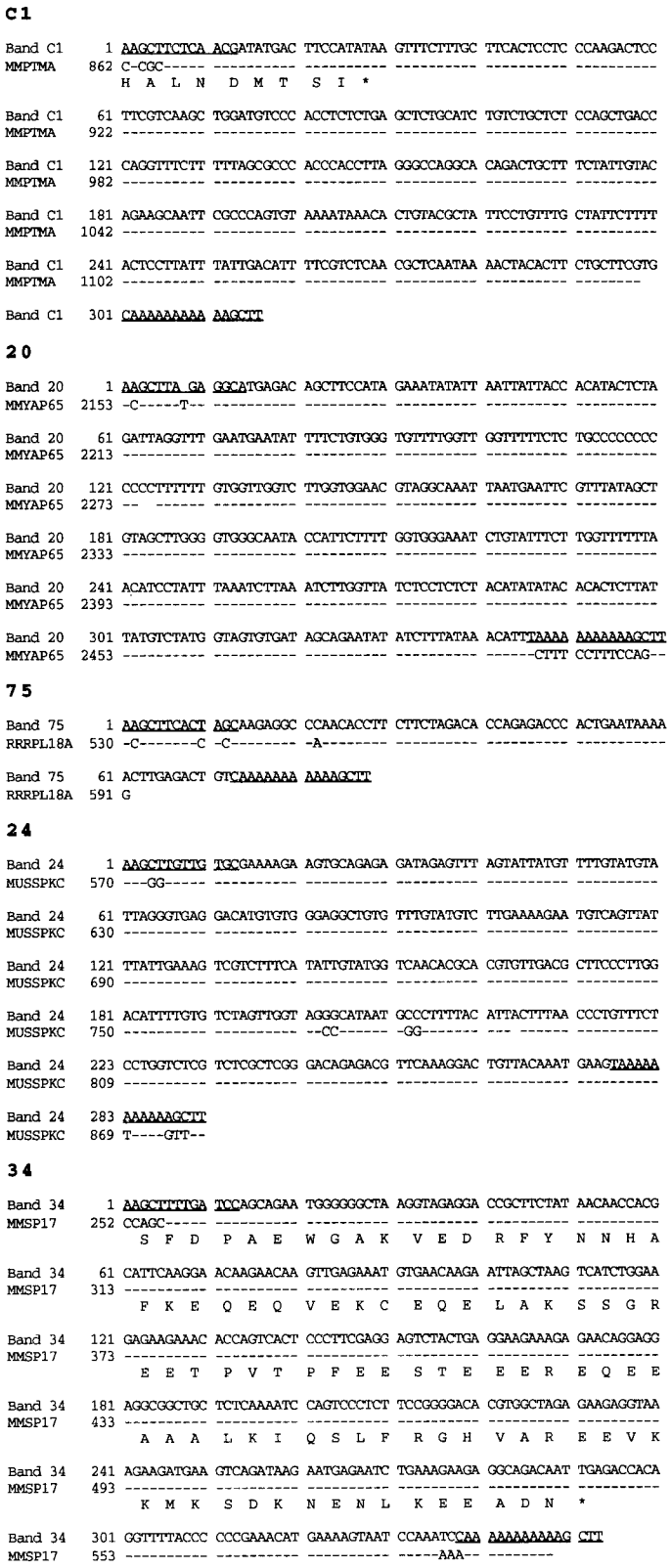

Fig. 3. Nucleotide sequence of 5 cDNA clones identified in differential display and homology with sequences in GenBank**.** Sequence comparison between band C1 and murine tropomyosin α (accession # X64831), band 20 and murine Yes-associated protein (YAP65, accession # X80508), band 75 and rat ribosomal protein L18α (accession # X14181), band 24 and murine KC (accession # J04596), band 34 and murine sperm antigen Sp-17 (accession # Z46299) was performed with BLAST. Primer sequences for PCR amplification are underlined.

TABLE II. Characterization of Differential Display Products in SCC

Clones	Size (bp)	Expression[a]					Genbank match
		BALB/c[b]	Pam 212	LY-1	LY-2	LU-1	
C1	316	+					Mouse Tropomyosin α
20	361		+	+	+	+	Mouse YAP65
75	88		+	+	+	+	Rat L18a
24	310			+	+	+	Mouse KC protein
34	353			+	+	+	Mouse Sp17

[a]Expression was scored based on relative signal intensities following short exposure of Northern blots.
[b]Primary keratinocyte from neonatal BALB/c mice.

The murine KC protein [18], a homologue of human growth-related protein (*gro*) α [30], belongs to a large family of C-X-C chemokines that includes human IL-8 [31,32]. *gro* family chemokines have functioned as potent chemoattractants for neutrophils [33,34], as growth factors for melanocytes and melanomas [35–38], and also may stimulate angiogenesis in cancer [39,40]. Constitutive expression of KC is found in certain tumor lines [41,42]. Interestingly, metastatic variants induced a marked infiltration of granulocytes and ulcerated early when compared with the parental Pam 212 line (C.W. Smith, unpublished observation). In the Pam 212 model, KC expression was significantly elevated in the reisolates, and showed minimal expression in primary keratinocytes and the parental tumor Pam 212 line. The functional significance of KC in tumorigenesis, and the potential relationship to tumor progression and metastasis observed in this study, remains to be determined, along with the basis for the constitutive expression of KC in reisolates, and whether KC protein may be functionally involved in SCC tumor progression.

YAP65 was first identified as a proline-rich phosphoprotein with a ligand that binds to the SH3 domain of the Yes proto-oncogene product [43]. Yes is one of several *Src*-related kinases, which form a family of non-receptor protein tyrosine kinases that associate with certain membrane receptors and downstream proteins, largely through SH2 and SH3 structural domains present in the amino-terminal half of the *Src* family members [44,45]. The *Src* family has been implicated in signal transduction pathways essential to cell structures, growth, differentiation, and oncogenesis [46]. Zhao et al., 1992 [47] demonstrated that tyrosine kinase activity of c-*Src* greatly increased during keratinocyte differentiation induced by calcium, while the tyrosine kinase activity of the Src family member c-Yes was inactivated in association with decreased Yes expression. The significance of the Yes-mediated signal pathway and the down-regulation of the Yes gene during keratinocyte differentiation is unknown. The interaction of YAP65 with c-Yes has been shown to occur via a novel 38 amino acid protein motif designated as the WW domain, based on conserved positioning of four aromatic amino acids, including two tryptophan residues [48]. The consensus binding site for WW domain was characterized as XPPXY [49], found in many other important proteins, such as the retroviral gag protein, sodium channels, formin, interleukin receptors, MAP kinase, and dystrophin [50]. The significance of WW domain and XPPXY-containing protein interactions has yet to be directly associated with human diseases. In our murine tumor model, full-length mRNA for YAP65 was detected in all tumor lines but not in keratinocytes which undergo terminal differentiation. We propose that YAP expression may be important for regulation of proliferation and differentiation in SCC, probably serving as a protein module bridging Yes with downstream signal pathway components.

L18a, a ribosomal protein, and ribosomal RNA are the main components of ribosomal subunits. Homeostasis of ribosomal proteins is maintained in normal cells for the controlled production of cellular proteins [51]. Certain carcinomas, such as colorectal carcinoma, overproduce a subset of ribosomal proteins [52–56]. Ribosomal proteins with mutations have also been detected as processed tumor-specific antigens recognized by helper T cells [57]. Our finding that ribosomal protein L18a is upregulated in SCC tumor cells supports the notion that overproduction of certain types of ribosomal proteins may be the consequence of transformation, or alternatively, expression of these ribosomal proteins may be associated with func-

tions related to cell growth or survival in neoplasia.

Little is known about Sp17, a protein antigen expressed primarily in the testes [19,58]. In SCC, expression of Sp17 is limited to metastatic variants. As such, its pattern of expression resembles other differentiation antigens detected in neoplasms using immunologic methods. We do not yet know if this molecule is expressed as a tumor-associated antigen in other SCC, or if expression is limited to the tumors of Pam 212 lineage. Such a molecule could be useful as a tumor marker for SCC.

The basis for expression of the combination of genes identified thus far in our study is unclear, but transcription factors that control expression of at least one of the candidates identified in this study have been implicated in the development of SCC. AP-1 transcription factors have been shown to play an important role in regulating expression of KC and other proinflammatory cytokines that constitute part of the early response to injury. Greenhalgh and Yuspa, 1988, [59] studied the murine squamous papilloma cell line 308 and demonstrated that transfection of the *fos* oncogene that binds the AP-1 element results in malignant conversion of this non-malignant cell line. The resulting cells formed tumors when injected subcutaneously in mice. Domann et al., 1994, [60] developed another malignant variant line from 308 by irradiation, designated 308–10Gy5, and found that this line exhibited a constitutive increase in AP-1 DNA binding and transactivating activities. In a separate set of experiments, the same authors stably transfected 308–10Gy5 and another chemically transformed murine squamous cell line with cDNA expressing a transdominant c-JUN mutant [61]. These transfected cells showed much-reduced tumor-forming potential compared to parental cells or cells transfected with vector only. These experiments demonstrate the significant role of AP-1 activity and related genes in malignant conversion in murine keratinocytes. The results using the current model system suggest the possibility that KC may be one target of AP-1 activity associated with tumor progression and metastasis.

In conclusion, we have found genes with diverse roles in cell growth and differentiation, providing evidence that complex mechanisms are involved in tumor progression. Application of a gene screening strategy such as DD has proven fruitful in our model system for studying molecular events involved in tumor SCC progression. By combining sensitivity and simplicity to amplify a spectrum of cDNAs with PCR, we have been able to simultaneously compare expression of mRNAs in cells representing different stages of transformation and tumor progression. Simultaneous comparison of mRNA expression among normal cells and multiple tumor cell lines with different neoplastic potential reduced the false positive recovery of DNA products and enhanced our ability to identify candidates with broad activities, which might be either up- or down-regulated. It should be possible to directly evaluate the function of the candidate genes in the model system. Furthermore, with the use of such a multistage model, chemopreventive or therapeutic agents can be designed and analyzed to target gene products associated with either cell transformation or tumor metastasis. Genes identified in our tumor model can also be readily evaluated as potential biomarkers for chemoprevention. For instance, elevated KC messenger RNA was present in metastatic variants of Pam 212. Increased KC protein production was also detected in the conditioned media of the same cell lines at ng/ml levels by ELISA [62]. Thus, KC protein and its human homologs such as gro-α and IL-8 may become suitable biomarkers for malignancy in squamous cell carcinoma.

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