Reactivation of Developmentally Expressed p63 Isoforms Predisposes to Tumor Development and Progression

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Abstract

Genes that are active during normal development are frequently reactivated during neoplastic transformation. We now report that developmentally expressed TAp63 isoforms are frequently reactivated in human squamous cell carcinomas. To determine the consequences of TAp63 reactivation, we induced TAp63α expression during chemically-induced skin carcinogenesis. Deregulated TAp63α expression dramatically accelerated tumor development and progression, frequently resulting in epithelial-mesenchymal transitions to spindle cell carcinomas and lung metastases. Consistent with this observation, we detected high levels of Twist and N-cadherin in tumors overexpressing TAp63α. Thus, as observed for other developmental pathways, aberrant reactivation of TAp63 predisposes to tumor development and progression. (Cancer Res 2006; 66(8): 3981–6)

Introduction

p63, a p53 homologue, is transcribed into six isoforms that contain (TA) or lack (∆N) a transactivation domain and is primarily expressed in stratified epithelia, such as the epidermis (1–3). Whereas p53 functions as a classical tumor suppressor gene (4), the role for p63 in tumorigenesis remains controversial. A recent study reported that p63−/− mice are predisposed to develop spontaneous tumors (5). In contrast, it was found that p63−/− mice from a different line (p63−/−; ref. 2) are not tumor prone (6). In addition, p63Rdn2/− mice are not predisposed to gamma irradiation-induced lymphomas (6, 7) or two-stage skin carcinogenesis (6), arguing against a role for p63 in tumor suppression. Consistent with this notion, p63 is rarely mutated in human cancers (6). Instead, squamous cell carcinomas (SCC) from different organs express high levels of p63 (6). Although the p63 isoform that is overexpressed was not determined in most studies, overexpression of both TA- and ∆Np63 isoforms has been documented (6). The expression of TAp63 in epithelial tumors is especially interesting because TAp63 isoforms function primarily during epidermal morphogenesis, whereas TAp63 levels are low in mature epidermis (1, 8). We previously found that deregulated expression of TAp63α in mature epidermis causes hyperproliferation and a failure of the epidermis to undergo terminal differentiation (9). Moreover, ectopic TAp63α expression in single-layered lung epithelia results in the development of proneoplastic squamous metaplastic lesions (9). Taken together, these data suggest that TAp63 could potentially function as a proto-oncogene in epithelial tissues.

We now report that developmentally expressed TAp63 isoforms were reactivated in the majority of well-differentiated head and neck SCC (HNSCC). To understand the biological significance of TAp63 reactivation, we used our previously established gene-switch TAp63α mouse model and found that deregulated expression of TAp63α in the epidermis accelerated chemical skin carcinogenesis and malignant progression. Furthermore, during the early stages of tumorogenesis, TAp63 induced two genes involved in tumor progression, Twist and N-cadherin. Taken together, our data show that developmentally expressed TAp63 isoforms function as proto-oncogenes when their expression is inappropriately reactivated.

Materials and Methods

Transgenic/knockout mouse lines. K14.Glp65 (activator) and TK.TAp63α (target) mice were previously described (9). K14.Glp65 mice and TK.TAp63α mice were crossed to give rise to binegeric K14.Glp65/ TAp63α mice (referred to as gene-switch TAp63α mice in this article).

Chemical carcinogenesis. Mice were shaved 2 days prior to initial treatment at 8 weeks of age and as needed throughout the study. Dimethylbenz(a)anthracene (DMBA; Sigma, St. Louis, MO) was applied at a single subcarcinogenic dose of 50 μg per mouse. RU486 treatment was initiated 2 days after DMBA treatment and mice were treated with 100 μg of RU486 thrice a week. 12-0-tetradecanoyl-phorbol-13-acetate (TPA; Sigma) was applied beginning 1 week after initiation with DMBA, 10 μg per mouse, once a week for the duration of the study. Mice were monitored weekly for tumor formation, size, and number. A total of 19 gene-switch TAp63α and 18 control (K14.Glp65) littermates were included in the two-stage chemical carcinogenesis experiments.

Immunofluorescence. Tumors were fixed in 10% neutral buffered formalin and immunofluorescence was done using antibodies guinea pig anti-K14 (9), rabbit anti-K1 (9), rabbit anti-K13 (10), mouse anti-N-cadherin (Zymed, San Francisco, CA), and rabbit anti-Twist (Santa Cruz Biotechnolog., Santa Cruz, CA). Secondary antibody conjugates used were Alexa-conjugated fluorochromes 594 goat anti-guinea pig, 488 goat anti-rabbit, and 488 goat anti-mouse (Molecular Probes, Eugene, OR).

Flow cytometry. Formalin-fixed paraffin-embedded tumors were sectioned into five 50-μm sections per sample. Sections were deparaffinized in xylene, rehydrated, and incubated overnight in 100 μg/mL of RNaseA in PBS. The following day, the cells were dissociated by stirring the samples in 5% trypsin for 2 hours at 37°C. The dissociated cells were fixed in cold ethanol and resuspended in 50 μg/mL propidium iodide. DNA content was measured on a Beckman-Coulter Epics XL-MCL. Per sample, 20,000 events were recorded and the data were analyzed using the Modfit 3.0 program (Verity Software House, Topsham, ME). The DNA index was calculated as the ratio of the mean channel position of the G0/G1 peak of the tumor sample and the mean channel position of the G0/G1 peak of normal control skin. The

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coefficients of variation were calculated using the formula method by dividing the peak width at the half-maximum height of the G0/1 peak by 2.35 (11).

Ras sequencing. Genomic DNA was isolated from seven lung metastases that developed in gene-switch TAp63α mice. H-ras was amplified using primers FW 5′-GGG GCA GGA GCT CTT GGA TGG CAC GCT and RV 5′-CCT GGA CTG GAT GAC TCT GTC GAA GGA CTT. The sequencing of PCR products was done by Lone Star Labs (Houston, TX).

Human HNSCC tumors. Thirty-six HNSCC tumors and case-matched tissue samples adjacent to the tumors were obtained from consenting patients at the Department of Otolaryngology, Oregon Health and Science University, as previously described (12). Part of the tissue samples were frozen and stored in liquid nitrogen immediately after removal; whereas the other part was formalin-fixed and processed for paraffin-embedding. After histologic analysis, 18 tumors were classified as well/moderately differentiated and 18 tumors were classified as poorly differentiated.

RNA extraction and real-time reverse transcription-PCR. Relative gene expression levels were determined by real-time reverse transcription-PCR. For mouse tumors, RNA from 16 gene-switch TAp63α (bigenic K14.Glp65/TAp63α) and 16 control (monogenic K14.Glp65) papillomas was extracted using TriZol (Invitrogen, San Diego, CA) or RNeasy mini kits (Qiagen, Chatsworth, CA), and cDNA was prepared using the High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA). RNA from human tumors was extracted using RNAzol-B (Tel-Test, Friendswood, TX), and further purified using RNeasy mini kits (Qiagen). Total RNA (5 μg) was treated with DNaseI (Ambion, Austin, TX), and subjected to a reverse transcription reaction using avian myeloblastosis virus reverse transcriptase (Roche). Assays-on-Demand TaqMan probes for Twist, N-cadherin, and 18S were obtained from Applied Biosystems. TaqMan Universal PCR Master Mix (Applied Biosystems) was used for PCR amplification of the cDNA. Real-time reverse transcription-PCR reactions were done in triplicate with a DNA Opticon 2 System (MJ Research, Waltham, MA). Each mRNA was normalized to the level of 18S in each sample and the relative amount of each gene was determined using the comparative C(T) method. For the human HNSCC samples, TA- or ΔNp63 were considered overexpressed if their relative expression level was >2-fold higher than the expression level in the adjacent mucosa. The Kruskal-Wallis test was used to determine whether levels of gene expression were statistically different between two groups. The Spearman correlation test was used to determine the correlation between expression levels of different genes in the same samples. P < 0.05 indicated that there is a statistically significant relationship between the two variables. All experiments involving mice were done under Institutional Animal Care and Use Committee approval (protocol AN-546).

Results and Discussion

Deregulated TAp63α expression accelerates skin carcinogenesis and tumor progression. To determine the consequences of reactivation of TAp63 expression for epithelial tumorigenesis, we exposed gene-switch TAp63α mice, in which TAp63α expression could be induced by topical application of RU486, to the two-stage chemical carcinogenesis protocol (13). In addition to initiation with DMBA and tumor promotion with TPA, mice were treated with RU486 thrice a week, which resulted in the induction and maintenance of TAp63α expression (see Fig. 3A). Mice carrying only the activator component of the gene-switch system (K14.Glp65) were used as controls for the carcinogenesis experiments. As shown in Fig. 1A, gene-switch TAp63α mice developed tumors at a faster rate than control littermates (P < 0.01). Moreover, gene-switch TAp63α mice developed significantly more tumors per mouse than control mice (Fig. 1B and D; P < 0.01). More strikingly, deregulated expression of TAp63α caused accelerated conversion to SCC (Fig. 1C and E; P < 0.01). Papillomas and SCCs were identified by gross appearance (Fig. 1D and E) and histologic examination (Fig. 2A). Furthermore, immunofluorescence using antibodies

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against K1, a marker for benign papillomas (14), and K13, a marker for malignant progression (10), was done on a subset of SCCs to confirm the histotype (Fig. 2B). Histologic analysis showed that ~50% of gene-switch TAp63α mice, but none of the control mice, had tumors that progressed to spindle cell carcinoma (SPCC), indicating that an epithelial-mesenchymal transition (EMT) had occurred (Fig. 2C; ref. 15). In addition, all gene-switch TAp63α mice had developed lung metastases by the time they were sacrificed (29-36 weeks after initiation), whereas none of the control mice developed lung metastases (Fig. 1F). Flow cytometric DNA content analysis was done on a total of 2 normal newborn skin samples and 11 papillomas, 11 SCCs, 4 SPCCs, and 7 lung metastases that developed in gene-switch TAp63α mice (D). None of the histograms showed evidence of DNA aneuploidy, suggesting that mechanisms other than genomic instability underlie the accelerated tumor progression in gene-switch TAp63α mice. Note that the control sample used was newborn skin, which has a higher S phase fraction than adult skin. S phase fractions for tumor samples ranged from 15% to 20%.

Figure 2. Deregulated TAp63α accelerates skin carcinogenesis and tumor progression. Gene-switch TAp63α and control mice were exposed to the two-stage chemical carcinogenesis protocol. Gene-switch TAp63α mice developed tumors at a faster rate than control mice and displayed accelerated conversion to SCC. The typical histologic appearance of a gene-switch TAp63α papilloma and a gene-switch TAp63α SCC in (A). Immunofluorescence using antibodies against K1 (green), a marker for benign lesions (B, left), and K13 (green), a marker for malignant progression (B, right) was done on consecutive sections of the same tumor. K14 staining (red) was used to highlight the epithelial component of the tumor. B, the conversion from a benign tumor to a malignant tumor. In addition to the accelerated conversion to SCC, ~50% of the gene-switch TAp63α mice developed SPCC, and all gene-switch TAp63α mice developed lung metastases (C). In contrast, none of the control mice developed SPCC or lung metastases. Flow cytometric DNA content analysis was done on a total of 2 normal newborn skin samples and 11 papillomas, 11 SCCs, 4 SPCCs, and 7 lung metastases that developed in gene-switch TAp63α mice (D). None of the histograms showed evidence of DNA aneuploidy, suggesting that mechanisms other than genomic instability underlie the accelerated tumor progression in gene-switch TAp63α mice. Note that the control sample used was newborn skin, which has a higher S phase fraction than adult skin. S phase fractions for tumor samples ranged from 15% to 20%.

Twist and N-cadherin are highly expressed in gene-switch TAp63α tumors. Because tumors that develop in gene-switch
TAp63α mice progress in the absence of genomic instability, we wanted to determine the molecular mechanism by which TAp63α accelerates tumor progression. We initially used Affymetrix microarrays to identify genes that are induced or repressed by TAp63α in papillomas and found that two genes that promote tumor progression through EMT, Twist, and N-cadherin (18, 19), were expressed at higher levels in gene-switch TAp63α papillomas than in control papillomas (Twist, 2.3-fold; N-cadherin, 2.1-fold). Coexpression of Twist and N-cadherin has previously been observed in diffuse-type gastric cancers (20), and Twist induces N-cadherin in Madin-Darby canine kidney cells (18). Surprisingly, we did not observe changes in the expression of E-cadherin, a target gene of Twist, which is regulated by Twist independently of N-cadherin (18). Although it has been postulated that loss of E-cadherin is a prerequisite for EMT, N-cadherin can promote EMT and tumor cell invasion even in the presence of functional E-cadherin (19).

To validate the changes in gene expression, we did real-time reverse transcription-PCR on 16 gene-switch TAp63α and 16 control papillomas. As predicted, TAp63 expression levels were higher in gene-switch TAp63α tumors when compared with control tumors (P < 0.05; Fig. 3A). In contrast, ΔNp63 expression levels were similar in control and gene-switch TAp63α tumors (P = 0.45; Fig. 3A). In addition, both Twist and N-cadherin were expressed at higher levels in gene-switch TAp63α papillomas than in control papillomas (P < 0.05; Fig. 3B), whereas changes in E-cadherin expression levels did not reach statistical significance (P = 0.66; data not shown). Furthermore, Twist and N-cadherin expression levels correlated with TAp63 expression levels (Spearman correlation, P < 0.01 for both Twist and N-cadherin; data not shown). Interestingly, the regulation of Twist and N-cadherin by TAp63 seems to be dependent on tissue context because these genes were not induced by TAp63α in primary keratinocytes (data not shown). To determine if these changes in gene expression also occur at the protein level, we did immunofluorescence analysis for Twist and N-cadherin. As predicted, we could detect Twist expression in gene-switch TAp63α, but not in control tumors (Fig. 3C, top). Interestingly, Twist was also expressed in the hyperplastic epidermis adjacent to gene-switch TAp63α tumors (Fig. 3C, bottom), suggesting that Twist induction is an early event in...
tumor progression in this mouse model. Furthermore, whereas both control and gene-switch TAp63α tumors expressed N-cadherin in the stroma, only gene-switch TAp63α tumors expressed N-cadherin in epithelial (i.e., K14 positive) cells (Fig. 3D).

Because N-cadherin may be a target gene of Twist (18), we hypothesized that TAp63α could activate the EMT signaling cascade by inducing Twist. However, using chromatin immunoprecipitation analyses on gene-switch TAp63α tumors, we were unable to show an interaction between TAp63α and the Twist promoter, suggesting that the induction of Twist by TAp63α is a secondary event. Alternatively, TAp63α could directly regulate Twist expression by a mechanism independent of direct DNA binding, as has been shown to occur at the MDR1 promoter (21).

Reactivation of developmentally expressed p63 isoforms occurs in human HNSCC. The ability of deregulated TAp63α expression to accelerate tumorigenesis in our mouse model prompted us to assess whether TAp63 expression is reactivated in human HNSCC. Similar to the epidermis, deregulated TAp63α expression in stratified epithelia of the oral cavity caused hyperplasia and hyperproliferation (Fig. 4A), suggesting that TAp63α performs similar functions in these different stratified epithelia.

To test whether TAp63 isoforms are reactivated during tumorigenesis, we determined the relative TA- and ΔNp63 expression levels in human HNSCC by performing real-time reverse transcription-PCR analysis. After normalizing gene expression levels to 18S, we found that of the 36 tumor samples, 14 had increased TAp63 expression levels (39%), whereas 18 had increased ΔNp63 expression levels (50%) when compared with the corresponding adjacent mucosa (Fig. 4B). Interestingly, TA- but not ΔNp63 expression was correlated with the clinicopathologic stage of the tumors (Fig. 4B). Of the 18 well/moderately differentiated HNSCC samples, 13 had increased TAp63 expression (72%) compared with 1 of the 18 poorly differentiated tumors (6%; P < 0.05; B). ΔNp63 was frequently overexpressed in both well/moderately (44%) and poorly differentiated tumors (56%; P = 0.89). Bars, median expression level of each group. TAp63 expression levels correlated with Twist and N-cadherin expression levels (C). Furthermore, there was a positive correlation between Twist and N-cadherin expression levels (C, bottom). All correlations were P < 0.05, indicating that a statistically significant relationship existed between the two variables.

Figure 4. Developmental TAp63 isoforms are reactivated in human HNSCC. Gene-switch TAp63α and control mice were treated with RU486 in the oral cavity. After 5 days of treatment, tongue epithelia were hyperplastic and hyperproliferative (A), showing that deregulated TAp63α expression has similar phenotypic consequences in various stratified epithelia. Eighteen well/moderately differentiated and 18 poorly differentiated HNSCC samples were analyzed for the expression of TA- and ΔNp63, Twist, and N-cadherin. After normalization to 18S, relative expression was determined by comparing the gene expression levels in the tumor to the gene expression levels in the adjacent uninvolved mucosa (UM). Of the 18 well/moderately differentiated tumors, 13 had increased TAp63 expression (72%), whereas only one of the poorly differentiated tumors overexpressed TAp63 (6%; P < 0.05; B). ΔNp63 was frequently overexpressed in both well/moderately (44%) and poorly differentiated tumors (56%; P = 0.89). Bars, median expression level of each group. TAp63 expression levels correlated with Twist and N-cadherin expression levels (C). Furthermore, there was a positive correlation between Twist and N-cadherin expression levels (C, bottom). All correlations were P < 0.05, indicating that a statistically significant relationship existed between the two variables.
Closely correlated with TAp63 expression levels (Fig. 4C) showed that both Twist and N-cadherin expression levels were associated with EMT and a consequent loss of expression of tumour progression. Furthermore, we found that tumor progression of advanced tumors that develop in our mouse model, we observed that TAp63 overexpression caused accelerated tumor progression. Furthermore, we found that tumor progression was associated with EMT and a consequent loss of expression of epithelial markers, including K14 and p63. In human cancers, however, the situation seems to be more complex because TAp63 loss in advanced tumors is sometimes, but not always, associated with a loss of other epithelial markers, such as ∆Np63 (22, 23). At present, it remains unclear whether loss of TAp63 expression in this subset of tumors is a cause or consequence of tumor progression. Another subset of human tumors, i.e., those that lose both TAp63 and ∆Np63 during tumor progression, may more closely mimic advanced tumors that develop in our mouse model. In this subset of tumors, induction of Twist and N-cadherin by TAp63 during the early stages of tumorigenesis may cause tumor progression through EMT. The loss of all epithelial markers during EMT suggests that, once the signaling pathways required for tumor progression become activated, an autoregulatory feedback loop may maintain the expression of these genes such that tumor progression can proceed in the absence of p63 expression. In summary, our data show that, analogous to other developmental pathways, aberrant reactivation of TAp63 predisposes to tumor development and progression, and may serve as a prognostic indicator in a subset of human cancers.

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22. Park BJ, Lee SJ, Kim JL, et al. Frequent alteration of Np63 during tumor progression, may more closely mimic advanced tumors that develop in our mouse model. In this subset of tumors, induction of Twist and N-cadherin by TAp63 during the early stages of tumorigenesis may cause tumor progression through EMT. The loss of all epithelial markers during EMT suggests that, once the signaling pathways required for tumor progression become activated, an autoregulatory feedback loop may maintain the expression of these genes such that tumor progression can proceed in the absence of p63 expression. In summary, our data show that, analogous to other developmental pathways, aberrant reactivation of TAp63 predisposes to tumor development and progression, and may serve as a prognostic indicator in a subset of human cancers.
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