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Activation of p21(CIP1/WAF1) in mammary epithelium accelerates mammary tumorigenesis and promotes lung metastasis

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ABSTRACT

While p21 is well known to inhibit cyclin-CDK activity in the nucleus and it has also been demonstrated to have oncogenic properties in different types of human cancers. *In vitro* studies showed that the oncogenic function of p21is closely related to its cytoplasmic localization. However, it is unclear whether cytoplasmic p21 contributes to tumorigenesis *in vivo*. To address this question, we generated transgenic mice expressing the Akt-phosphorylated form of p21 (p21T145D) in the mammary epithelium. The results showed that Akt-activated p21 was expressed in the cytoplasm of mammary epithelium. Overexpression of Akt-activated p21 accelerated tumor onset and promoted lung metastasis in MMTV/*neu* mice, providing evidence that p21, especially cytoplasmic phosphorylated p21, has an oncogenic role in promoting mammary tumorigenesis and metastasis.

1. Introduction

The serine/threonine protein kinase Akt, also known as protein kinase B (PKB), plays a critical role in tumorigenesis by regulating a variety of cellular processes, including preventing cells from undergoing apoptosis [1,2]. The importance of Akt in promoting cell survival was demonstrated by a study showing that targeted expression of constitutively activated Akt to the mammary epithelium of transgenic mice significantly delayed the mammary involution with impaired apoptosis [3]. A number of Akt/PKB downstream targets have been indentified which are associated with apoptotic functions, such as NF-κB[4], Bad[5], Forkhead transcription factors [6,7], MDM2 [8–10] and p21 [11].

p21(CIP1/WAF1) is a well-known universal inhibitor of cyclin-CDKs, which mediates cell cycle arrest [12–14]. However, it has also been revealed to be a multifaceted protein that it has an "oncoprotein"-like property in addition to its role as a tumor suppressor. Increased p21 expression was shown to correlate with tumorigenesis and poorer prognosis in different types of human cancers, including brain, prostate, ovarian, cervical, breast and esophageal squamous cell cancers [15].

One of the mechanisms in which p21 exerts its oncogenic function is through protecting cells from apoptosis through interaction with and inhibition of pro-apoptotic caspases (procaspase 3, caspase 8 and 10) or apoptosis-regulating kinases (SAPKs and ASK1) in the cytoplasm [16–18]. These observations indicate that the functions of p21 are closely related to its cellular localization, as nuclear p21 holds cell cycle inhibitory activity, while the cytoplasmic p21 possesses an anti-apoptotic oncogenic function. Therefore, the mechanisms that regulate the shuttling of p21 between cytoplasm and nucleus become important to cancer biology. Studies have shown that Akt can phosphorylate p21 at threonine 145 (T145), resulting in stabilization and cytoplasmic localization of p21 [11,19,20]. The cytoplasmic localization of p21 has an increased resistance to apoptosis [21]. More importantly, the cytoplasmic localization of p21 and overexpression of phosphorylated p21 at T145 are associated with worse overall survival in breast cancer patients [22].

However, the oncogenic function of cytoplasmic p21 was largely studied in the *in vitro* culture system. To directly study the role of cytoplasmic p21 in mammary development and tumorigenesis, we generated transgenic mice expressing the Akt-phosphorylated form of p21 (p21T145D) in the mammary epithelium. The results showed that Akt-activated p21 expressed in the cytoplasm of mammary epithelium. Overexpression of Akt-activated p21 accelerated the tumor onset and promoted lung metastasis in

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MMTV/*neu* mice, providing evidence that p21, especially the cytoplasmic p21 has an oncogenic role in promoting mammary tumorigenesis and metastasis.

2. Materials and methods

2.1. Generation of transgenics

The cDNA encoding p21D (T145D) was subcloned from PCDNA3 vector into the p206 vector [3,10]. Generation of transgenic mice was described previously [3]. The genotypes of transgenic mice were identified by transgnene specific PCR and further confirmed by Southern blot analysis described previously [3]. Genotypes of mice from MMTV/*neu* crosses performed by transgnene specific PCR at DNA Core facility in M.D. Anderson Cancer Center.

2.2. Histological analysis

Complete autopsies and gross and microscopic examinations of tissues were performed. Histological analysis was performed on the lower left mammary fat pad tissues or mammary gland tumor tissues as previously described [3]. Whole-mount preparation was prepared from the lower right mammary fat pad as previously described [3].

2.3. Immunohistochemistry

Immunohistochemistry was performed as previously described with the following exceptions [3]. Primary antibody used was a rabbit polyclonal anti-p21 (C-19; 1:50 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

2.4. Tissue harvesting and immunoblottings

Tissues from mammary gland or tumor were flash frozen in liquid nitrogen and transferred to −80 °C for storage. Protein lysates were prepared and immunoblots were performed as previously described with the following exceptions [23]. Anti-E-cadherin immunoblot analyses were performed on 100 μg of total protein lysate with rabbit polyclonal anti-E-cadherin antibody (1:1000 dilution; Abcam, Cambridge, MA.).

2.5. Reverse transcription and quantitative PCR

RNA was extracted from frozen mammary gland tissues using the RNeasy Lipid Tissue Midi kit (Qiagen). Reverse transcription was performed and quantitative PCR was performed in M.D. Anderson DNA Core Facility with primers (p21 Forward CGA CTG TGA TGC GCT AAT GG; p21 Reverse TCT CGG TGA CAA AGT CGA

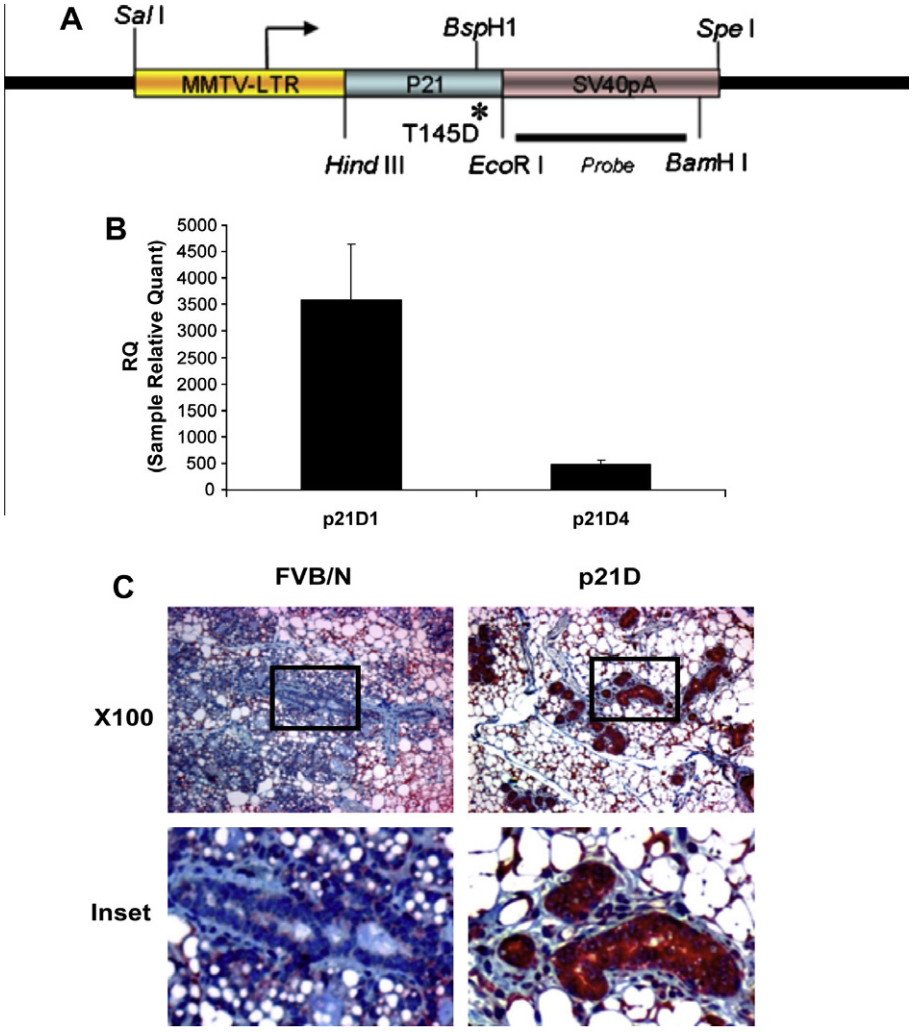

Fig. 1. Generation and identification of transgenic mice. (A) Structure of the MMTV/p21D transgene. (B) Quantitative RT-PCR analysis of p21D transcript expression in two MMTV/p21D strains. Mammary glands from three 7-day-pregnant mice of each strain were analyzed in triplicate and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. (C) Immunohistochemical analysis of p21 expression on mammary glands from FVB/n control (left column) and MMTV/p21D mice (right column) pregnant for 7 days. Inset shows magnification of indicated area.

AGT TC; p21 Probe 5′FAM-TCC AGG AGG CCC GTG AGC GAT-BHQ1) that only recognize the human p21 transgene.

3. Results and discussions

3.1. Generation of MMTV/p21D transgenic mice

To study whether Akt-mediated p21 phosphorylation affects mammary development and contributes to the development of mammary tumors, we generated transgenic mice that express the Akt-phosphorylated form of p21 (p21D) in mammary epithelium. p21D mimics the active phosphorylated state of p21 by Akt, which was generated by substituting the threonine residue at 145 with aspartic acid. To accomplish this, the full length cDNA of p21D was placed under the MMTV promoter and microinjected into FVB/N mouse zygotes (Fig. 1A). Genotypes of transgenic mice were identified by transgene-specific PCR and further confirmed by Southern blot analysis with a probe directed to the SV40 component of transgene (data not shown). The expression of the transgene was examined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay from mammary glands of mice that were 7 days pregnant (Fig. 1B).The primers used for the RT-PCR recognize only the human p21 transgene but not the endogenous p21 gene (data not shown and Section 2.5). The tissue specificity of transgene expression was confirmed by immunohistochemical staining on sections of mammary epithelium from mice

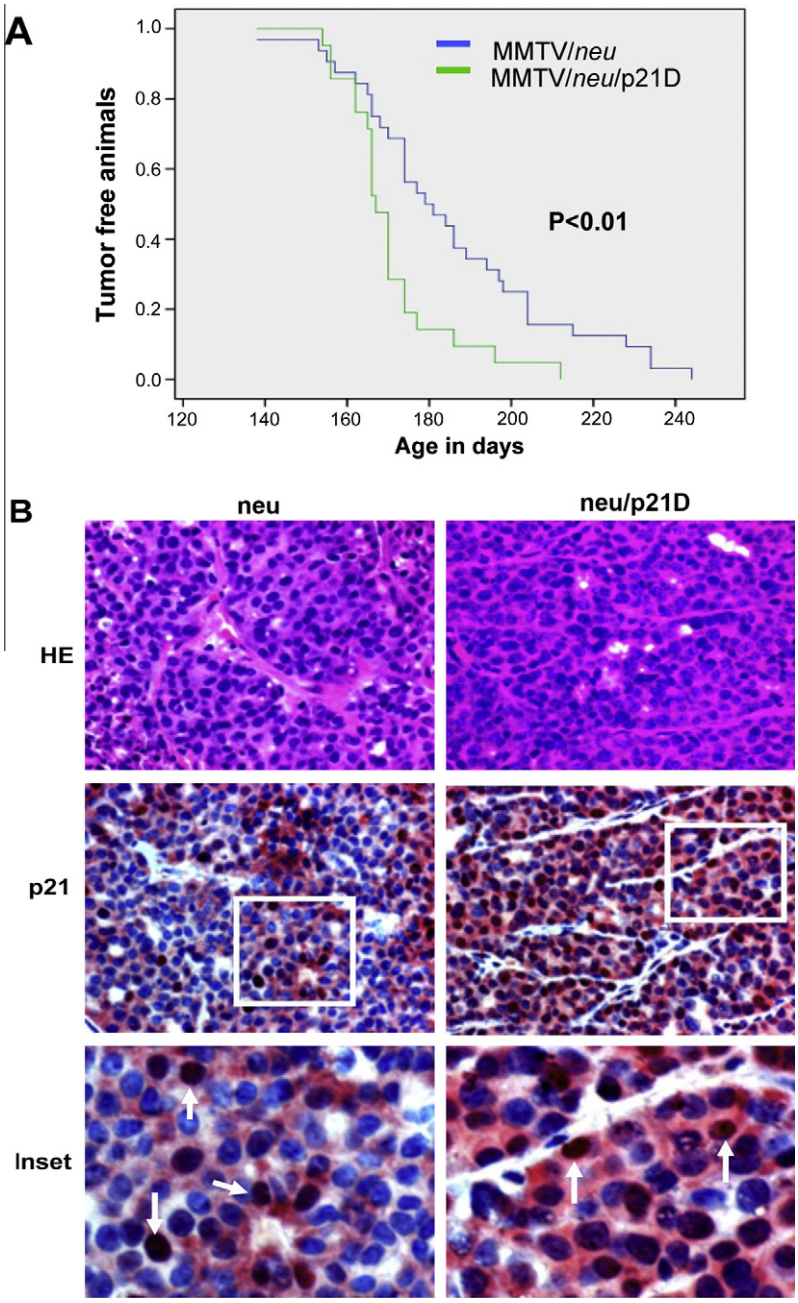

Fig. 2. Expression of p21D accelerated tumor onset in MMTV/*neu* mice. (A) Mammary tumor kinetics of MMTV/*neu*/p21D and MMTV/*neu* mice, $p < 0.01$ by Log Rank (Mantel-Cox) test. (B) Representative histological patterns of MMTV/*neu* and MMTV/*neu*/p21D tumors (top). Immunohistochemical analysis of p21 expression in mammary tumors from MMTV/*neu* (middle row, left column) and MMTV/*neu*/p21D (middle row, right column) mice. Red color represents p21 cytoplasmic staining. White arrows show examples of p21 staining in the nucleus. Inset shows magnification of indicated area. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

at the same stage. The expression of p21D was uniformly expressed in the mammary epithelium, predominantly in the cytoplasm (Fig. 1C). The red color for the p21 staining represents the cytoplasmic staining of p21. Under the same conditions, endogenous p21 was not detected in the parental FVB/N mice. The result was consistent with previous studies, which indicated that Akt-mediated p21 Thr145 phosphorylation was critical for the translocation of p21 to the cytoplasm [11,22].

3.2. Coexpression of p21D and neu results in accelerated tumor formation

To explore the role of p21D in mammary tumorigenesis, a cohort of virgin female MMTV/p21D mice were monitored for tumor formation. None had developed mammary tumors after a year of observation.

We next examined whether p21D expression could collaborate with other oncogenes to promote mammary tumorigenesis. To accomplish this, we generated bitransgenic mice, coexpressing p21D and activated *neu* in the mammary epithelium, by interbreeding MMTV/*neu* mice [24] with MMTV/p21D mice. Cohorts of virgin female bitransgenics and MMTV/*neu* mice were monitored for tumor formation. The results revealed that expression of p21D in mammary epithelium accelerated the tumor onset in MMTV/*neu* mice (Fig. 2A). 50% of the bitransgenic mice showed tumor formation at 167 days ($n = 21$) as compared with 179 days ($n = 32$) from MMTV/*neu* mice ($p < 0.01$).

To confirm that the accelerated tumor formation in MMTV/*neu*/p21D bitransgenic mice was due to the transgene expression of p21D, immunohistochemical staining was performed on mammary tumor samples. The results revealed that expression of p21D was detected in bitransgenic mammary tumors. Consistent with the study in MMTV/p21D mice, p21D was also expressed in the cytoplasm in addition to the nucleus (red color represents p21 staining in the cytoplasm. The black nuclei are labeled by white arrows showing examples of p21 staining in the nucleus) of tumor cells (Fig. 2B). Please notice that there was more p21 cytoplasm staining observed in the MMTV/*neu*/p21D bitransgenic mice. The MMTV/*neu* mice also demonstrated cytoplasmic staining, though weaker than that of the bitransgenic mice. It is expected as AKT, which can phosphoylate p21 and enhance cytoplasmic localization, is activated in the MMTV/neu mice. These observations suggest that p21D accelerates the induction of *neu* mammary tumors.

Considering the fact that phosphorylation of p21 by Akt leads to cytoplasmic translocation of p21, our results suggest that cytoplasmic p21 may acquire a gain-of-function in promoting tumorigenesis. Consistent with what we found, several studies also have demonstrated an oncogenic function of p21. For example, p21 knockout mice showed delayed development of thymic lymphomas via a sensitized apoptotic response mechanistically independent of p53 [25,26]. Additionally, the cyclin-binding motif of p21 was showed to have a direct tumorigenic role in an oligodendroglioma mouse model [27]. Taken together, these studies indicate that in addition to its tumor suppression function, p21 might also have an oncogenic function.

3.3. p21D promoted metastasis in MMTV/neu transgenic mice

To further explore whether the expression of p21D affects mammary tumor metastasis, MMTV/*neu* and MMTV/*neu*/p21D bitransgenic mice were sacrificed when the tumor burden reached 15 mm in diameter. Lung metastasis was examined by microscopic observations on H&E stained sections from lung tissue. The results showed that 61% of the tumor-bearing MMTV/*neu*/p21D bitransgenic mice ($n = 23$) developed lung metastasis, which were about an approximately 2-fold increase ($p < 0.05$) in the penetrance of the metastatic phenotype as compared to the 33% metastasis in

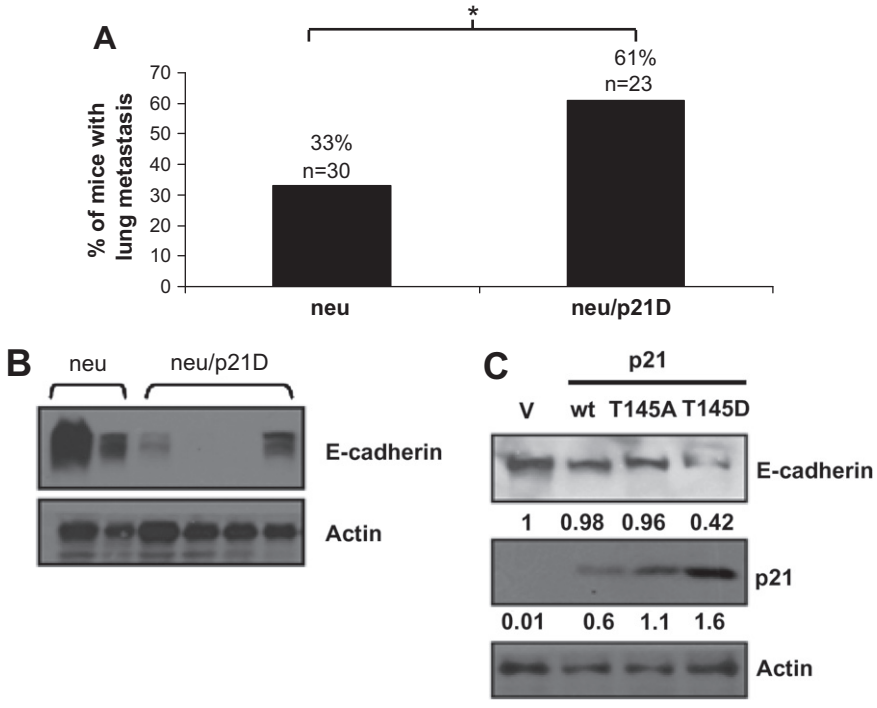

Fig. 3. p21D promoted metastasis in MMTV/*neu* transgenic mice. (A) Percentage of mammary tumors bearing mice with lung metastasis when the tumor burden reached 15 mm in diameter. *, *Chi* sqaure test demonstrated a significant difference between occurrence of metastasis in MMTV/*neu* and MMTV/*neu*/p21D mice ($p < 0.05$). (B) Immunohistochemical analysis of E-cadherin expression in mammary tumors from MMTV/*neu* and MMTV/*neu*/p21D mice. (C) Lysates of 293 cells were transfected with vector control, wild type p21, p21T145A or p21T145D and analyzed by immunoblotting of E-cadherin.

parental MMTV/*neu* transgenic mice ($n = 30$) (Fig. 3A). This result suggested that p21D promotes the metastasis of *neu* mammary tumors.

To further explore the possible mechanism of the oncogenic role of p21 in metastasis we examined the expression of E-cadherin, which mediates cell-cell adherences and whose loss is often associated with the increased motility and invasiveness of tumor cells. The results showed that expression of E-cadherin was decreased in p21D expressing mammary tumors compared to that in MMTV/*neu* tumor tissues (Fig. 3B). In addition, decreased expression of E-cadherin was observed in p21T145D transfected 293 cells, while no significant changes of E-cadherin expression were observed in either wild type p21 or p21T145A transfected cells (Fig. 3C). Our results suggest that p21 inhibits E-cadherin, which might contribute to the enhanced metastatic potential. Further studies will be needed to explore the detailed mechanism of how p21D down-regulates E-cadherin.

Taken together, this study elucidates the importance of p21 activation by Akt in a physiological context by using a mammary gland specific transgenic mouse model. The results here show that overexpression of Akt activated p21 accelerated the tumor onset and promoted lung metastasis in MMTV/*neu* mice, providing evidence that p21, especially the cytoplasmic p21, has an oncogenic role in promoting mammary tumorigenesis and metastasis.

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