

Tumor suppressor gene alterations of spontaneously malignant transformed cells from human embryonic muscle *in vitro*

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Abstract. Recent research has shown that mesenchymal stem cells (MSCs) which were cultured for long time could transform malignantly, the transformation mechanism is not clear yet, it might be associated with the activation of oncogenes and inactivation of tumor suppressor genes. In our initial investigation, we found that the cells arising from human embryonic muscle could spontaneously transform into malignancy *in vitro* and we obtained 6 immortalized cell lines. In this study, polymerase chain reaction (PCR) was used to assay several tumor suppressor genes of these cell lines, and homozygous deletions within chromosomal band 9p21 including *MTAP* (methylthioadenosine phosphorylase), *p16* and *p15* were detected. PCR products of *p53* exons 7 and 8 of these novel tumor cell lines were assayed by sequencing, and the results showed high prevalence of mutations in these regions, the mutation rate reached as high as 8% in exon 7 and 14% in exon 8, and all of them were point mutations, the intron 7 changed more significantly, including piece deletion, insertion, frameshift and point mutation, it showed almost no similarity to that of the *wt p53* sequence, that was totally different from other *p53* mutation data published. All the mutation sequences were identical in 6 cell lines, this suggest that there may be a common mutation mechanism and strong selective advantage in these novel tumor cell lines over long-term culture. In conclusion, our research shows that the inactivation of tumor suppressor genes may play an important role in the process of malignant transformation of embryonic muscle cells *in vitro*.

Introduction

The development of tumors is generally accepted to be a multistep process in which alterations in oncogenes and tumor suppressor genes play an important role (1,2). The process of carcinogenesis involves the gain of oncogene activity and the loss of tumor suppressor gene function, such as *Rb*, *p53* and cyclin-dependent kinase inhibitor (*CDKI*) family (3-5). The aberrant gene could interfere in many cell functions, such as cell proliferation, differentiation and apoptosis. The accumulative mutations constitute the base of cell malignant transformation in gene level. The acquired capabilities of tumor cells include their ability to proliferate continuously ignoring apoptosis or growth-inhibitory signals, generating their own mitogenic signals.

At present, the reports on the malignant transformation of human cell *in vitro* are rare. Recent research has found that mesenchymal stem cells (MSCs) cultured for long time *in vitro* could lead to malignant transformation, but the mechanism is not very clear. The activation of oncogenes and inactivation of tumor suppressor genes are considered to be one of the mechanisms (6-9). We have found that human embryonic muscle cells which were cultured *in vitro* long-term could become spontaneously immortalized, and we obtained 6 novel malignant cell lines, this new type of cell lines were named human embryonic muscle-derived malignant transformed cells (hEMTCs). These hEMTCs could be passaged stably and showed characteristics of sarcoma *in vivo* (10).

Herein, we report several tumor suppressor gene alterations of hEMTCs (from 5 different genetic backgrounds) and we demonstrate homozygous fragment deletions in chromosomal bands 9p21 and extensive mutations of *p53* gene.

Materials and methods

Cell culture. Artificial abortive embryos were obtained from Chaozhou Central Hospital, and embryonic use was ratified by the Ethics Committee of Chaozhou Central Hospital, and the informed consent of the mother and/or their family was signed, embryos ranged from 7 to 13 weeks. Leg muscle tissue was cut into 0.5-mm³ pieces, put into 24-well plates, and

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Table I. Sequence of PCR primers.

Gene	Primer sequence	PCR conditions	Product size (bp)
<i>p16</i> Exon 1	F: 5'-CGGAGAGGGGGAGAGCAGGCA-3' R: 5'-GCGCTACCTGATTCCAATTC-3'	95, 60, 72°C, each for 1 min	277
<i>p16</i> Exon 2	F: 5'-TTCCTTTCCGTCATGCCGG-3' R: 5'-GTACAAATTCTCAGATCATCAGTCCTC-3'	95, 57, 72°C, each for 1 min	394
<i>p16</i> Exon3	F: 5'-CCCGCTTTCGTAGTTTTTCAT-3' R: 5'-TTATTTGAGCTTTGGTTCTG-3'	95, 58, 72°C, each for 1 min	355
<i>p15</i> Exon 1	F: 5'-TTTCCCAGAAGCAATCCAGGCGCG-3' R: 5'-CGATCTAGGTTCCAGCCCCGATCC-3'	95, 58, 72°C, each for 1 min	494
<i>β-actin</i>	F: 5'-TGGCACCACACCTTCTACAATGAGC-3' R: 5'-GCACAGCTTCTCCTTAATGTCACGC-3'	95, 59, 72°C, each for 1 min	396
<i>p53</i> Exon (7-8)	F: 5'-AGGTTGGCTCTGACTGTACC-3' R: 5'-CTTGTCCTGCTTGCTTACCTC-3'	95°C 1 min, 57°C 1 min, 72°C 1.5 min	610
<i>MTAP</i> Exon 2	F: 5'-ATTGGAATAATTGGTGGAACAGGC-3' R: 5'-CCAGCAACAGAATGAGAAGTGAT-3'	95, 57, 72°C each for 1 min	341
<i>MTAP</i> Exon 3	F: 5'-CAGTCTACCATCAGAGTTCCC-3' R: 5'-TTGCAAGGAGGACGCAAT-3'	95, 57, 72°C each for 1 min	342
<i>MTAP</i> Exon 4	F: 5'-CTCTAGGAGAAAACAGTTGGTG-3' R: 5'-GACCAGCTACAATAGCCTAAAG-3'	95, 57, 72°C each for 1 min	275
<i>MTAP</i> Exon 5	F: 5'-GACCTAGATAAAGTTGACTC-3' R: 5'-TACACCTTCCAGAAAGACTA-3'	95, 57, 72°C each for 1 min	220
<i>MTAP</i> Exon 6	F: 5'-AGTTGTGCATGTGCTAGTAT-3' R: 5'-ACCCATGCTATATGTGCTTA-3'	95, 57, 72°C each for 1 min	328
<i>MTAP</i> Exon 7	F: 5'-AGTTCTAGTAACCTCCAGTG-3' R: 5'-CTACAGACATGCCTGATTGT-3'	95, 57, 72°C each for 1 min	195
<i>p21</i> (RT-PCR)	F: 5'-CAGGGGACAGCAGAGGAAGA-3' R: 5'-GGGCGGCCAGGGTATGTAC-3'	95, 59, 72°C each for 1 min	335
<i>p53</i> (RT-PCR)	F: 5'-ACCACCATCCACTACAACACTACAT-3' R: 5'-GCAAGCAAGGGTTCAAAGAC-3'	95, 57, 72°C each for 1 min	589

cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% FCS (Lanzhou Minhai Bio., Co.), 100 µg/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml fluconazole in a 37°C humidified, 5% CO₂ air. When cultured cells became confluent, 0.25% trypsin with 0.02% EDTA was used for passage. After culturing for 4-7 months, the embryonic muscle-derived cells spontaneously transformed in 6 cell lines, and they came from 5 different genetic backgrounds (5 embryos). These hEMTCs could be passaged stably and immortalized. They were named MS0812, MS0504, M3, M4, M5B and M5C, respectively. M5B and M5C were from the same embryo but transformed malignantly in different culture time. The characteristics of line MS0812 was described clearly in our previous report (10). MS0504, M3, M4 and M5C could lead to fibrosarcoma in nude mice. M5B could lead to fibrosarcoma and rhabdomyosarcoma in nude mice which was confirmed by pathology (data not shown).

PCR for p16, p15, MTAP and p53. Genomic DNA was isolated from hEMTCs using a genomic DNA isolation kit (Universal Genomic DNA Extraction Kit Ver. 3.0, Takara Biotechnology Dalian Co., Ltd.) according to the manufacturer's instructions. The primers for PCR were synthesized in Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. PCR was performed for exons 1-3 of the *p16* (*CDKN2A*), exon 1 of *p15* (*CDKN2B*), exons 2-7 of *MTAP* (methylthioadenosine phosphorylase), exons 7-8 of *p53* in a KP-TC48 thermal cycler (HybriBio Ltd.). PCRs were carried out in 10 mM Tris (pH 8.6), 50 mM KCl, 1.5 mM MgCl₂, 0.4% DMSO, 0.2 mM dNTPs, 0.4 mM each primer, 50 ng of genomic DNA, and 5 unit of Taq polymerase (Takara Taq™, Takara Biotechnology Co., Ltd.) per 50-µl reaction. The PCR conditions and primer sequences are shown in Table I. Primers for β-actin were used as a positive control for the presence of DNA. Electrophoresis of

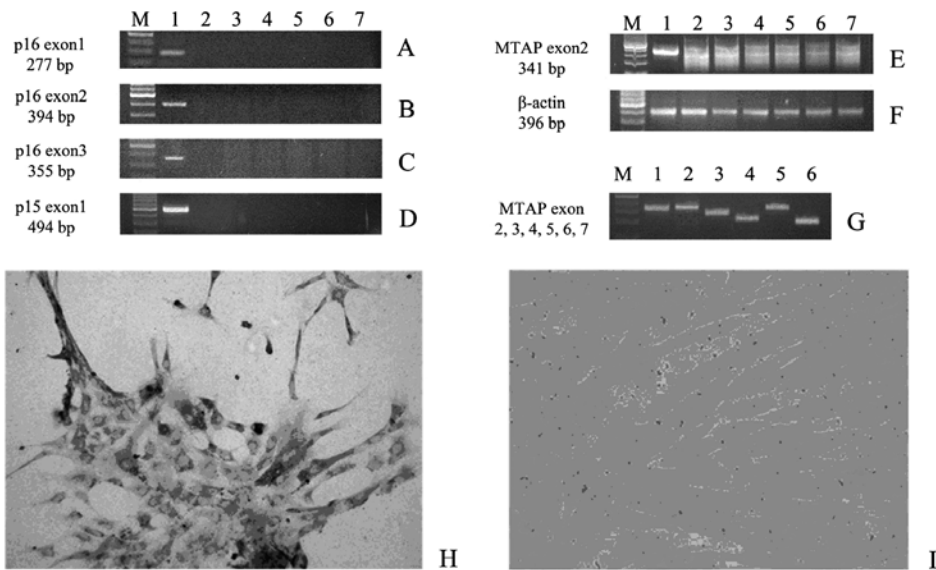


Figure 1. Interstitial deletions of 9p21. (A) Homozygous deletion of the *p16* exon 1, (B) exon 2, (C) exon 3 and (D) *p15* exon 1 in 6 hEMTCs. (E) PCR products of exon 2 of *MTAP*. (F) The internal control of β -actin. Lanes 1-8 from left to right, 100 bp ladder, the normal control, MS0812, MS0504, M3, M4, M5B, M5C (A-F). (G) Positive control of *MTAP*: lanes 1-7 from left to right, 100 bp ladder, exons 2-7 of *MTAP* in normal human embryonic muscle cells. (H) *p16* protein expression. The normal embryonic muscle cells were positive for *p16* staining in cytoplasm. (I) hEMTCs (M3, standing for 6 cell lines) were negative for *p16*.

PCR products was performed on a 2% agarose gel subsequently stained with ethidium bromide and photographed.

RT-PCR for *p21* and *p53*. Total RNA was isolated using TRIzol Reagent (Invitrogen) following the recommendations of the manufacturer. cDNA from each cell line was synthesized from denatured RNA (65°C for 5 min) by incubation at 42°C for 60 min with final quantities or concentrations of 3 μ l of 100 ng/ μ l oligo (dT), 2 μ l of 50 U/ μ l StrataScript reverse transcriptase, 1 μ l of 40 U/ μ l RNase Ribonuclease inhibitor (Takara, Dalian), and 4 μ l 10 mM dNTP mixture. Primers used for *p21* and *p53* of RT-PCR are summarized in Table I. β -actin was used for RT-PCR control. All RT-PCRs were carried out in a 50X PCR reaction containing 0.4 mM each primer, 5 units of Takara Taq, 1.5 mM MgCl₂, 4 μ l 10 mM dNTP Mixture. The reaction conditions are shown in Table I.

DNA sequencing. The PCR products of exons 7, 8 and intron 7 of *p53* and RT-PCR products of *p21* were sequenced directly by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (11). The sequence equipment was ABI PRISM 3730. BigDye terminator was used for sequence reagent. All samples were tested by simultaneous bi-directional sequencing (Table II).

Immunocytochemistry. MS0812, MS0504, M3, M4, M5B and M5C were cultured in 3.5-cm dishes, and at 80-90% confluence, they were fixed with 4% paraformaldehyde for 15 min, followed by washing in phosphate-buffered saline (PBS) 3 times, each 2 min, endogenous peroxidase activity were quenched in 3% H₂O₂ for 15 min, then were permeated in 0.25% Triton X-100 for 15 min. The anti-*p16* antibody (Zymed, Beijing) was applied at 1:50 dilutions in PBS and incubated in a humidity chamber at room temperature for 2 h.

The secondary antibody staining kit was SP-9000 of broad spectrum provided by the Zhongshan Jinqiao Company, Beijing. AEC served as chromagen. Next, the samples were observed under a powerful optical microscope (x40).

Results

Homozygous deletions within chromosome bands 9p21 which included *p16*, *p15* and *MTAP*. To assess the status of *p16* in our cell lines, we used PCR assays in which *p16* exons 1-3 fragments were amplified from genomic DNA. No *p16* (including exons 1-3) specific product was detected in the 6 hEMTCs. However, specific PCR products using genomic DNA from normal embryonic muscle cells were detected (Fig. 1A-C). *p15* and *MTAP* also locate at chromosome bands 9p21, and their status were assessed, the results showed that *p15* exon 1 (Fig. 1D) and exons 3-7 of *MTAP* (data not shown) were deleted in the 6 cell lines, while the normal control showed specific products (Fig. 1G). Nevertheless, an amplifications of *MTAP* exon 2 showed several non-specific bands in the 6 hEMTCs, but the positive control of normal embryonic muscle cells showed only one specific band in the same reaction system (Fig. 1E). β -actin was used for internal control and could be detected in all PCRs from the 6 cell lines (Fig. 1F). We performed immunostaining for *p16* and found out that the 6 hEMTCs were negative (Fig. 1I), the normal embryonic muscle cells showed positive staining in the cytoplasm (Fig. 1H).

Sequence of *p53* exons 7, 8 and intron 7. The PCR products of *p53* exons 7, 8 and intron 7 were successfully amplified, and we sequenced the 6 hEMTCs (MS0812, MS0504, M3, M4, M5B and M5C) and found that *p53* in the 6 hEMTCs had a wide range of change, and the mutations were complicated. All 6 hEMTCs exhibited the same mutation

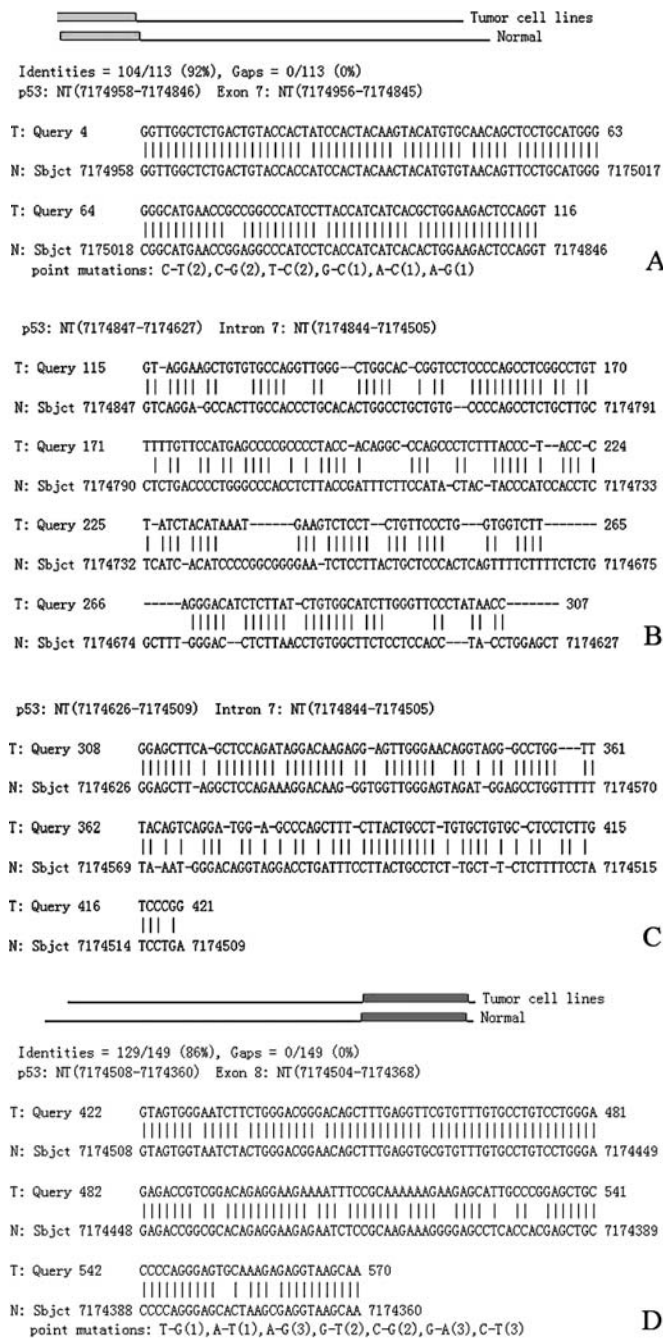


Figure 2. The sequence of *p53* in 6 hEMTCs (NT 7174958-7174360). (A) sequence of *p53* exon 7 (NT 7174958-7174846). (B and C) sequence of *p53* intron 7 (NT 7174847-7174509). (D) Sequence of *p53* exon 8 (NT 7174508-7174360).

model. Exons 7 and 8 of *p53* which compared to the *wt p53* in Genebank (<http://www.ncbi.nlm.nih.gov/>) showed high frequency of mutations which reaching as high as 8% (Fig. 2A) and 14% (Fig. 2B), respectively. All of them were point mutations. The intron 7 changed more significantly, including piece deletion, insertions, frameshift and point mutations, showed almost no similarity to the *wt p53* sequence, and we could only use BLAST (Basic Local Alignment Search Tool) for comparing part of the intron 7 sequence (from NT 7174626 to 7174509) (Fig. 2C), and the rest (from NT 7174847 to 7174627) was aligned manually

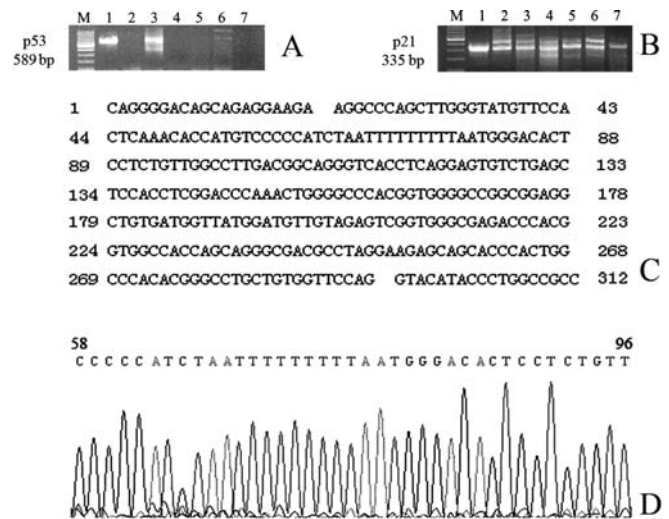


Figure 3. The transcription of *p21* and *p53* in 6 hEMTCs. (A) RT-PCR products of *p53* and (B) *p21* in 6 hEMTCs (lanes 2-8 from left to right, normal human embryonic muscle cells, MS0812, MS0504, M3, M4, M5B and M5C). (C) *p21* cDNA sequence and (D) sequence mapping of line M3 ranged from NT 27510435 to NT 27510573; NT 27511778 to NT 27511973 in Genebank.

since the structure was changed significantly and BLAST could not be aligned (Fig. 2D).

p53 and *p21* of RT-PCR and DNA sequencing of *p21*. We assayed the transcription of *p53* in 6 hEMTCs and only M3 showed the *p53* transcription (Fig. 3A). The transcription of *p21* in the 6 hEMTCs was also assayed using the RT-PCR. All 6 hEMTCs showed transcription product of *p21* but with a non-specific band of about 450 bp in line MS0812, MS0504, M3, M4, M5B (Fig. 3B), while the normal control showed only one specific band in the same PCR conditions (Fig. 3B). The RT-PCR products of *p21* in 6 hEMTCs and normal control were sequenced, and only M3 could be sequenced totally. The expected length was 335 bp, and the real length was 312 bp, the sequence was totally changed and had no similarity to the *wt p21* (Fig. 3C), and the sequence signal was specific (Fig. 3D). However, the control (normal human embryonic muscle cells) was the same as *wt p21* sequence (data not shown). The other 5 hEMTCs could only be sequenced partly, and the sequenced 180 bp was about the same as corresponding M3 (data not shown). The *p21* (RT-PCR) sequence of line M3 (Fig. 3C) exhibited sequential nucleotide 'T' and rich 'GC' regions, these special structures could lead to the weak sequence signal which make the sequencing impossible. Therefore, we got one complete result.

Discussion

At present, the reports on malignant transformation of cultured human cells *in vitro* are rare (6-9,12,13), while malignant transformation *in vitro* could be observed in embryonic cells from mice (14-16). One hypothesis to explain this phenomenon is that the number of mutational events



Location (codon)	Nucleotide substitution	Mutation effect	Frequency	Mutation recorded
Exon 7				
231	C→T (ACC→ACT)	Thr (synonymous)	7	No
235	C→G (AAC→AAG)	Asn to Lys (missense)	0	No
238	T→C (TGT→TGC)	Cys (synonymous)	4	No
240	T→C (AGT→AGC)	Ser (synonymous)	9	No
244	C→G (GGC→GGG)	Gly (synonymous)	21	No
248	G→C (CGG→CGC)	Arg (synonymous)	3	No
249	A→C (AGG→CGG)	Arg (synonymous)	6	No
252	C→T (CTC→CTT)	Leu (synonymous)	8	No
256	A→G (ACA→ACG)	Thr (synonymous)	4	No
Exon 8				
262	T→G (GGT→GGG)	Gly (synonymous)	3	No
264	A→T (CTA→CTT)	Leu (synonymous)	7	No
268	A→G (AAC→GAC)	Asn to Asp (missense)	0	No
272	G→T (GTG→GTT)	Val (synonymous)	11	No
282	G→T (CGG→CGT)	Arg (synonymous)	7	No
283	C→G (CGC→CGG)	Arg (synonymous)	10	No
287	G→A (GAG→GAA)	Glu (synonymous)	8	No
289	C→T (CTC→TTC)	Leu to Phe (synonymous)	4	Infrequent
291	G→A (AAG→AAA)	Lys (synonymous)	4	No
293	G→A (GGG→GAA)	Gly to Glu (missense)	0	In skin cancer
295	C→A (CCT→CAT)	Pro to His (missense)	2	Infrequent
296	C→T, A→G (CAC→TGC)	His to Cys (missense)	0	In skin cancer
297	A→C, C→G (CAC→CCG)	His to Pro (missense)	2	In skin cancer
303	C→T (AGC→AGT)	Ser (synonymous)	2	No
304	A→G, T→A (ACT→GCA)	Thr to Ala (missense)	4	In skin cancer
306	C→A (CGA→AGA)	Arg (synonymous)	2	No

Frequency, the mutation frequency on this codon location which has been published. Mutation recorded, this mutation type which has been described in UMD *p53* database (<http://www.umd.be:2072/>).

required to confer immortality on rodent cells is fewer than the number required for human and avian cells (or the rates of mutation are different) (17). Consequently, no one human or avian cell can acquire all of the alterations necessary for indefinite growth before the onset of the crisis period (17). Recent research has shown that mesenchymal stem cells from human (6-8) or mice (18-20) which were cultured for long-term *in vitro* could transform malignantly, the mechanism underlying it is not clear yet. It might be associated with the activation of oncogenes and inactivation of tumor suppressor genes, such as the amplification of *c-myc* and the inactivation of *CDKI* family (6,8,14,21). We found that human embryonic muscle cells cultured *in vitro* long-term could become immortalized spontaneously, and have obtained 6 novel cell lines (10). This type of tumor cell lines expressed actin, vimentin and desmin *in vitro*, which suggest that these malignant cells were originated from embryonic muscle cells, and these cell lines could develop sarcoma in nude mice (10). However, malignant transformation

mechanism in gene level is not clear yet. The goal of this study was to determine if several classic tumor suppressor genes such as *p53*, *p21* and *p16* have an effect on the malignant transformation of embryonic muscle cells.

The interstitial deletions of the 9p21 were observed in a variety of tumor types, including acute lymphoblastic leukemia, glioma, melanoma, lung cancer, head and neck cancer, mesothelioma, ovarian cancer and bladder cancer (22-25). As *CDKI* family (including *p16*, *p15*, and *p21*) play the predominant role in both *p53* and *pRb* pathways, the inactivation of these tumor suppressor genes in this region, also including *MTAP* (methylthioadenosine phosphorylase), contributes to the malignant process in all these different tumor types (26,27). *CDKN2A* generates several transcript variants which differ in their first exons, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase (p16). The remaining transcript (*CDKN2A^{ARF}*; also called *p14*) is an alternate open reading frame (ARF) that specifies a protein which is structurally unrelated to the

products of the other variants. Because deletion of the *p16* and *p14* causes dysregulation of the *p53* and *pRb* pathways in most cancers, loss of *MTAP* activity is thought to be incidental and not of pathogenic consequence (28). However, in certain cancers, loss of *MTAP* has been observed in cells that retain *p16*. In a study of non-small cell lung cancer, homozygous deletion of *MTAP* occurred in 38% (19 of 50) of the samples compared with only 18% (9 of 50) for *p16* (30). The fact that *MTAP* is lost independently of *p16* hints that loss of *MTAP* may have some functional basis in tumor biology (29-31).

We assayed the status of tumor suppressor genes *p16*, *p15* and *MTAP* which located at 9p21 in the hEMTCs, and all 3 genes were deleted in these cell lines. This suggested that the interstitial deletion of 9p21 existed in this new type of sarcoma cell line as reported of adult human spontaneous transformed stem cells (6). The PCR products of exon 2 of *MTAP* showed some non-specific amplification and weak purpose band, this suggested that there may be a breakpoint near this location. This result was partly consistent with the report that a frequent breakpoint took place between *MTAP* exon 4 and exon 5 (30).

The *p53* tumor suppressor gene is mutated in over 50% of human cancers, 74% of these mutations are missense, which result in full-length, albeit mutant, proteins. This fraction of missense mutations is much higher than in other tumor suppressor genes (32) and implies that *p53* mutant proteins confer some selective advantage in carcinogenesis. It has been shown previously that mutant *p53* can act as an immortalizing gene when cotransfected into primary rat embryo fibroblasts along with a selectable marker (17), but whether this protocol works in human embryonic cells is not clear yet. Human adult spontaneous transformed stem cells were identified with *p53* inactivation by RT-PCR, but detailed information on *p53* was not documented (6). To determine whether a mutation at the *p53* locus is a common event in the pathways leading to spontaneous cellular immortalization, 6 malignant transformed cell lines were examined for *p53* alterations by PCR and DNA sequencing. We checked the mutations of our cell lines on the UMD *TP53* mutation database (<http://www.umd.be:2072/>). This is one of the two most regularly updated databases for *TP53* mutations, the other one is the International Agency for Research on Cancer (IARC) database (<http://www.p53.iarc.fr/index.html/>). Our results were totally different from the *p53* mutation database published (30-33). In particular, the sequence of intron 7 of *p53* in our hEMTCs is completely altered, this is different from the mutation type of *p53* which is mainly point mutation in malignant cells (37-42). The alterations of *p53* codon region will definitely change the transcription and expression of *p53* protein in hEMTCs. The new gain of function of mutant *p53* protein was able to play a role in the malignant transformation process. All our sequencing results of *p53* (exons 7, 8 and intron 7) in 6 cell lines show high consistency which suggested that a common mutation mechanism and strong selective advantage over long-term culture. The transcription of *p53* and *p21* in 6 hEMTCs was also tested using the normal primer sequence (Table I), and only M3 showed *p53* transcription. The negative results in the other 5 hEMTCs indicated no *p53* transcription, the

reason for this was not clear, *p53* complicated alterations may account for this negative result, as indicated by exons 7, 8 and intron 7 sequencing results.

We sequenced all the products (normal control and 6 hEMTCs) and found out that the sequence of the 6 hEMTCs was aberrant comparing with the *wt p21* sequence of normal control (335 bp, from NT 27510435 to NT 27510573; NT 27511778 to NT 27511973). We obtained one complete sequence of 312 bp from line M3 in which the sequence mapping was specific and credible (Fig. 3C and D). The reason why the transcription of *p21* changed so much needs further research.

In summary, spontaneous hEMTCs *in vitro* exhibit the interstitial deletions of the 9p21 which include *MTAP*, *p16* and *p15*, *p53* also changed significantly, the mutation mode in exons 7, 8 was point mutation, while intron 7 aberration was complex, including point mutations, fragment deletions and insertions. Our sequencing results of 6 hEMTCs (including 5 different genetic backgrounds) showed high identity in mutation mode, which suggests this mutation mode is common in our hEMTCs and has a strong selective advantage over long-term culture. The aberration and inactivation of tumor suppressor genes which include *p53*, *p21*, *p16*, *p14*, *p15* and *MTAP* permits DNA-damaged cells to continue replicating, this frequently contributes to the accumulation of gene mutations and plays an important role in the malignant transformation process.

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