Original Article

Experimental Study on Malignant Transformation of Human Bronchial Epithelial Cells Induced by Glycidyl Methacrylate and Analysis on its Methylation*

WANG An Na[1,2], WANG Quan Kai[1,2], YANG Min[1], HU Jie[1], DONG Lin[1], and XU Jian Ning[1,2,#]

1. National Institute of Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention, Beijing 100050, China; 2. Key Laboratory of Chemical Safety and Health, Chinese Center for Disease Control and Prevention, Beijing 100050, China

Abstract

Objective To establish the model of human bronchial epithelial cells (16HBE) malignant transformation induced by glycidyl methacrylate (GMA) and define the different methylation genes at different stages.

Methods DNA was extracted at different 16HBE malignant phases and changes of genes DNA methylation at different stages were detected using Methylation chip of 'NimbleGen HG18 CpG Promoter Microarray Methylation'. Methylation-specific PCR (MSP) was used to observe the methylation status of some genes, and then compared with the control groups.

Results The result showed that GMA induced 16HBE morphorlogical transformation at the dose of 8 μg/mL, and cell exposed to GMA had 1 374 genes in protophase, 825 genes in metaphase, 1 149 genes in anaphase, respectively; 30 genes are all methylation in the 3 stages; 318 genes in protophase but not in metaphase and anaphase; 272 genes in metaphase but not in protophase and anaphase; 683 genes in anaphase but not in metaphase and protophase; 73 genes in protophase and metaphase but not in anaphase; 67 genes in protophase and anaphase but not in metaphase; 59 genes in metaphase and anaphase but not in protophase.

Conclusion The pattern of DNA methylation could change in the process of 16HBE induced by GMA.

Key words: DNA methylation; Chip; Glycidyl methacrylate; 16HBE

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INTRODUCTION

Human may be in danger of exposing to low dose of chemical. Glycidyl methacrylate (GMA) is an important chemical widely used in resin, coating, adhesive, and plastic industries. The purpose of this study was to investigate the methylation in human bronchial epithelial cells malignant transformation induced by glycidyl methacrylate.

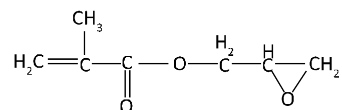

Figure 1. The chemical structure of GMA.

In the late 1980s, we firstly reported that GMA had mutagenicity in China, and found that GMA showed evident genotoxic and epigenotoxic effects in various systems *in vivo* and *in vitro*, including

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#Correspondence should be addressed to XU Jian Ning, professor, PhD, Tel: 86-10-83132592, E-mail: jnx999@263.net

Biographical note of the first author: WANG An Na, female, 1985, MD, majoring in toxicology, E-mail: oh_wanganna@126.com

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primary DNA damage, chromosomal damage, and gene mutations. We also found that GMA could induce malignant transformation in several types of mammalian or human cells.

Results from our previous research showed that GMA is a new mutagen and can induce neoplastic transformation of BALB/c 3T3 cells *in vitro*, thus, we suggested the potential carcinogenicity of this compound. In the previous researches of 16HBE exposed to GMA, we found that GMA could induce cell mutation and malignant transformation, and the early genetic effects of GMA were mainly on bacteria reverse mutation, DNA damage, cell cycle and apoptosis in cultured human bronchial epithelial cells (16HBE). The malignant transformation model of 16HBE induced by GMA was also successfully established *in vitro*. This research system provides a potential tool for the study of cell and molecular mechanism in the multistage carcinogenesis of this chemical. In the researches of 16HBE induced by GMA, we also found that GMA affected the function of the genes by damaging the cell chromosome. The defect of DNA repair system induced by GMA and the imbalance of injure- repair led to the cell transformation. And on the other hand, the changes of genes and pathways were also studied.

Up to date, the molecular mechanism of 16HBE malignant transformation induced by GMA is still unclear, so the purpose of this study was to find the pattern of DNA methylation changed in the process of 16HBE malignant transformation induced by GMA. Different from the classical genetic theory, DNA methylation plays an important role in the process of cells malignant transformation, gene regulation, cell proliferation, gene function and so on[1-4]. It was also considered to be the new tumor molecular biological markers (biomarker). In order to provide new evidences of carcinogenic characteristics of GMA, an epidemiologic monitoring and mechanism study of its chemical carcinogenesis was conducted in the present study.

MATERIALS AND METHODS

Chemicals

GMA (The purity ≥98.5%, Dow) was obtained from GUANG ZHOU SHUANGJIAN TRADING CO., LTD., the others were culture medium (Hyclone), trypsin (Amresco), Quick-gDNA™ MiniPre (Zymo, America), $EDTANa_2$ and DMSO (Sigma), NaCl, KCl, NaOH and so on (Beijing, China).

Cell

Human bronchial epithelial cells (16HBE) were obtained from California University.

Animal

The nude mice, Balb/c, weight 16-18 g, were purchased from Vital River Laboratory Animal Technology Co., Ltd.

Culture and Transformation

The 16HBE were cultured with minimum essential medium supplemented with 4 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. The cells were cultured exponentially, plated at the density of 5.0×10^5 and exposed to GMA at the dose of 8 μg/mL for 72 h. Same volume of DMSO was used as solvent. Then the cells were washed with PBS, detached by trypsinization and then plated to another culture bottle for another two GMA exposures at same dose and for same time period respectively. Subsequently, the GMA induced cells were signed for the first generation and cultured with medium of 5% FBS till the 30th generation. All the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. They were subcultured till cells grown more than 90% using 0.25% trysin.

The cells agglutination activity with low concentration of conA and agglutination time induced by GMA were observed. Because the transformed cells with anchorage independence could grow in semi-solid agar and showed a dose-reaction relationship with the concentration of GMA, we collected cells at the 10th, 20th, and 30th generation. Then the malignant transformation model of 16HBE induced by GMA *in vitro* was established. During the culture, the cells were identified by the tests of conA, colony forming frequency on soft agar, scanning electron microscope and tumorigenesis in nude mice. Test of immunocytochemical detection was also applied to confirm the derivation of cell and tumor formation.

Methylation Chip

The changes of DNA methylation at different stages were detected using the methylation chip of 'NimbleGen HG18 CpG Promoter Microarray Methylation'.

Genomic DNA Extraction and Fragmentation

Genomic DNA (gDNA) was extracted from 6 cells using a DNeasy Blood & Tissue Kit (Qiagen, Fremont, CA). The purified gDNA was then quantified and quality assessed by nanodrop ND-1000. Subsequently, the gDNA was sonicated to 200-1000 bp with a Bioruptor sonicator (Diagenode) and were agarose analysed including the sheared DNA.

Immunoprecipitation

One μg of sonicated genomic DNA was used for immunoprecipitation using a mouse monoclonal anti-5-methylcytosine antibody (Diagenode). Firstly, DNA was heat-denatured at 94 °C for 10 min, and then rapidly cooled on ice, followed by immunoprecipitating with 1 μL primary antibody and rocking agitation in 400 μL immunoprecipitation buffer (0.5% BSA in PBS) overnight at 4 °C. To recover the immunoprecipitated DNA fragments, 200 μL of anti-mouse IgG magnetic beads were added and incubated for another 2 h at 4 °C with agitation. After immunoprecipitation, a total of five immunoprecipitation washes were performed with ice-cold immunoprecipitation buffer. Washed beads were resuspended in TE buffer with 0.25% SDS and 0.25 mg/mL proteinase K for 2 h at 65 °C and then allowed to cool down to room temperature. MeDIP DNA were purified using Qiagen MinElute columns (Qiagen).

Whole Genome Amplification (WGA)

The MeDIP-enriched DNA was amplified using a WGA kit from Sigma-Aldrich [GenomePlex® Complete Whole Genome Amplification (WGA2) kit]. The amplified DNA samples were then purified with QIAquick PCR purification kit (Qiagen).

DNA Labelling and Array Hybridization

The purified DNA was quantified using a nanodropx ND-1000. For DNA labelling, the NimbleGen Dual-Color DNA Labeling Kit was used according to the manufacturer's guideline in the NimbleGen MeDIP-chip protocol (Nimblegen Systems, Inc, Madison, WI, USA). One μg DNA of each sample was incubated for 10 min at 98 °C with 1 OD of Cy5-9mer primer (IP sample) or Cy3-9mer primer (Input sample). Then, 100 pmol of deoxynucleoside triphosphates and 100 U of the Klenow fragment (New England Biolabs, USA) were added and the mixture was incubated at 37 °C for 2 h. The reaction was stopped by adding 0.1 volume of 0.5 mol/L EDTA, and the labelled DNA was purified by isopropanol/ethanol precipitation. Microarrays were hybridized at 42 °C for 16 h with Cy3/5 labelled DNA in Nimblegen hybridization buffer/hybridization component A in a hybridization chamber (Hybridization System - Nimblegen Systems, Inc., Madison, WI, USA). Following hybridization, washing was performed using the Nimblegen Wash Buffer kit (Nimblegen Systems, Inc., Madison, WI, USA). For array hybridization, Roche Nimblegen's CpG Promoter array was used, which is a single array design containing all known CpG Islands annotated by UCSC and all well-characterized RefSeq promoter regions (from about -800 bp to +200 bp of the TSSs) totally covered by 385 000 probes.

Methylation-specific PCR (MSP)

According to the result of chip, we selected some genes and then detected by MSP during malignant transforming stages of 16HBE induced by GMA according to the NucleoSpin® Tissue and EZ DNA Memylation-Gold Kit™, Zymo Research. High purity DNA was extract from the group of GMA and DMSO at different stages. Total DNA content was quantified by UV absorbance value measured at A260 and A280, and diluted to an adequate concentration.

The band expanded with methylation-specific PCR primers corresponding to the DNA methylation in the promoter region was marked as 'M'. The band expanded with non-methylation-specific primers was marked as 'U'.

RESULTS

Characterization of Human Bronchial Epithelial Cells Transformed by Glycidyl Methacrylate

No significant toxicity was observed when the cells were treated with GMA at the dose of 1 to 8 μg/mL for 72 h. But the treatment of 16 μg/mL GMA resulted in obvious cell death and low cloning formation (Figure 2).

The cells at the 14th generation in test group started to agglutinating in the conA solution, and at the 20th generation, it became more faster than the control group (Figure 3a, 3b). The transformed cells with anchorage independence could grow in semi-solid agar and showed a dose-reaction relationship with the concentration of GMA (Table 1), but cells in control group couldn't be agglutinated in the conA solution. The 30th generation transformed cells with anchorage independence could grow in semi-solid agar (Figure 3c, 3d).

The transformed cells could form subcutaneous tumor in nude mice. The tumor was diagnosed as squamous cell carcinoma in morphology confirmed by histopathology examination. Immunocyto chemical detection suggested that expression of cytokeratin (CK) was found in both 16HBE cells and tumor formed in nude mice (Figure 4). The transformed cells induced by GMA could form subcutaneous tumor in nude mice after inoculating after two weeks and grow rapidly to form a subcutaneous tumor. On the 21st d, the size of tumor reached 14.8-22.3 mm which were on mice oxter and cervix and had clear boundary. Whereas, no tumor was observed in the control group (DMSO-treated). The result of histopathology examination showed that it was the squamous cell carcinoma and there was no difference between the male and female mice. The oncocyte arranged nested as different shapes and few cells could be observed as adenoid. Nests of cancer cells had different sizes, but most were large cells. Parts of cytoplasm cancer cells were empty, which was hyaline cells; cancer tissue sheet necrosis and haemorrhage could be seen commonly in the cancer interstice.

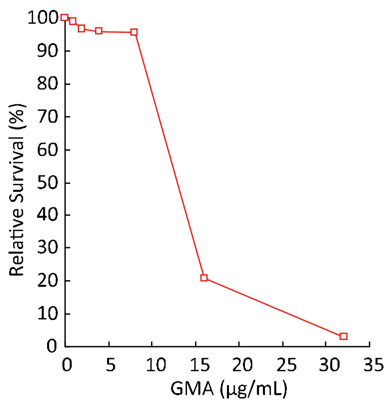
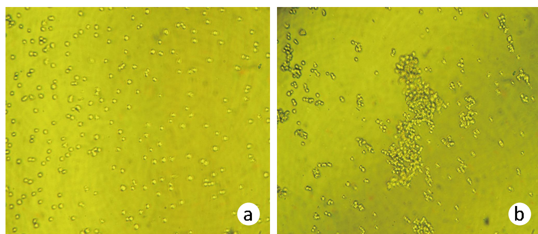
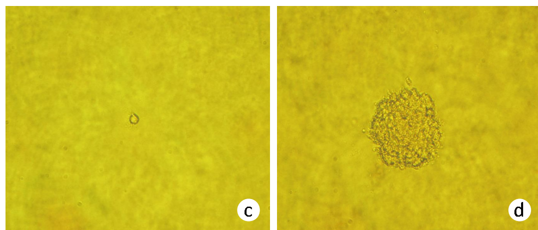


Figure 2. Relative survival rate of cells treated with GMA.



Transformed cells agglutinate in the conA solution (×100)



Anchorage independence of transformed cells (×100)

Figure 3. Transformed cells agglutinate in the conA solution (×100) and anchorage independence of transformed cells (×100). a: non-agglutinated (DMSO); b: agglutinated (GMA); c: no colony; d: colony.

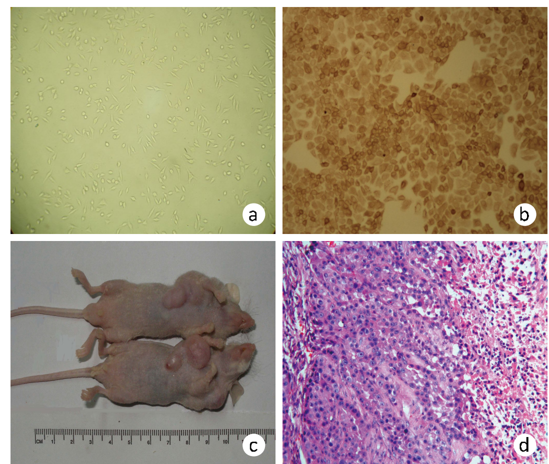


Figure 4. Tumorigenicity in the nude mice by GMA. a: Negative CK epression of 2BS cells; b: Positive CK epression of 16HBE cells; c: Tumorigenicity in the nude mice; d: Tumor pathological section in the nude mice (HE×400).

Table 1. Agglutination Time of Cells in the conA Solution (min)

Group	ConA (μg/mL)					
	6.25	12.5	25	50	100	200
DMSO (14th generation)	>30	>30	>30	25’34’’	22’54’’	22’33’’
GMA (14th generation)	>30	>30	25’54’’	21’07’’	15’47’’	10’24’’
DMSO (20th generation)	5’59’’	7’06’’	7’14’’	8’10’’	9’06’’	9’12’’
GMA (20th generation)	3’35’’	3’52’’	6’36’’	7’30’’	8’47’’	8’56’’

Result of Methylation Chip

The result of methylation chip was shown in Table 2 and Figure 5. As compared to the DMSO group, the GMA group had 1 374 methylation genes in protophase, 825 methylation genes in metaphase, 1 149 methylation genes in anaphase, 1 149 methylation genes in the three stages, 318 genes in protophase but not in metaphase and anaphase, 272 genes in metaphase but not in protophase and anaphase, 683 genes in anaphase but not in metaphase and protophase, 73 genes in protophase and metaphase but not in anaphase, 67 genes in protophase and anaphase but not in metaphase, and 59 genes in metaphase and anaphase but not in protophase.

The Alteration of DNA Methylation during Malignant Transformation of 16HBE Cells Induced by GMA

According to the results mentioned above, we selected *P15*, *OPCML,* and *THBS1* as biomarkers to detect the genes alteration by MSP during the malignant transformation of 16HBE cells induced by GMA. The results showed that the abnormal methylation in CpG island of *OPCML* could be considered as a specific gene in the process of 16HBE malignant transformation; *THBS1* could be considered as an early sensitive index; and *P15* could be considered as an cell malignant relative biological index (Figure 6).

Table 2. Change of Gene DNA Methylation at Different Stages

Methylation Occurred in Stages	Number of Methylation Genes	Genes (top 5)
Potophase	1 374	AADAT, ABCB6, ABCD3, ABHD15, ACAP2
Metaphase	825	A2BP1, AADAT, ABCA8, ABHD3, ABHD5
Anaphase	1 149	AADAT, ABCA10, ABCA8, ABCB1, ABCB10
Only protophase	318	ABCD3, ACD, ACVR2B, AFF1, AFF4
Only metaphase	272	A2BP1, ABL1, ACTN4, ACVR1B, ADAMTS7
Only anaphase	683	ABCA10, ABCB1, ABHD10, ABLIM1, ACOT12
Protophase and metaphase	73	ABHD8, ADAMTS14, ADAMTS2, AFG3L1, AGAP29
Protophase and anaphase	67	ACOXL, BCAR1, C20orf201, C4orf38, CCDC8
Metaphase and anaphase	59	ACOXL, BCAR1, C20orf201, C4orf38, CCDC8
Potophase, Metaphase, and Anaphase	30	AADAT, ABHD3, ABHD5, AKR7A3, AOX1

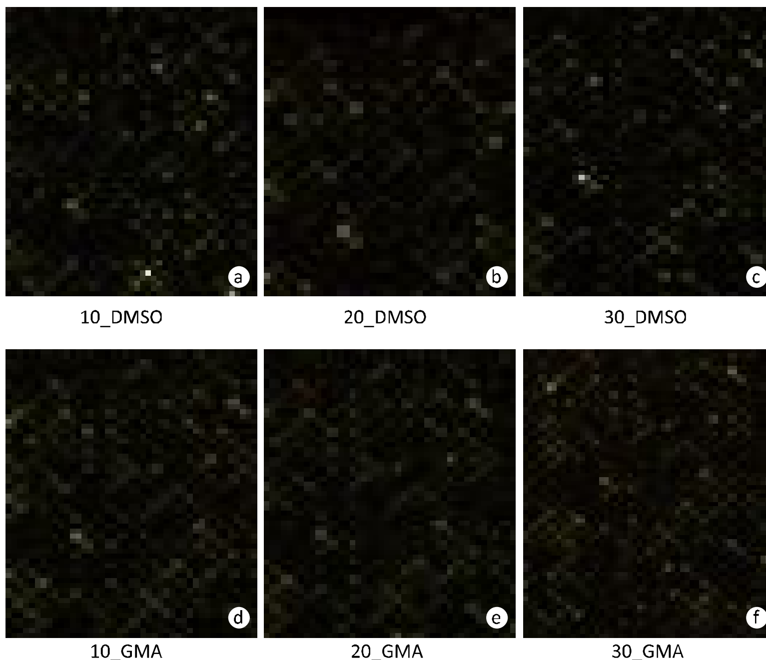


Figure 5. Scan of methylation chip at different stages in GMA and DMSO group. a: 10_DMSO; b: 20_DMSO; c: 30_DMSO; d: 10_GMA; e: 20_GMA; f: 30_GMA.

DISSCUSSION

Numbers of previous studies reported that GMA had genotoxicity, mutagenicity and cell transformation effect[5-8]. 16HBE, an SV40 large T antigen-immortalized human epithelial cell line, retains the parents' phenotypes with different lineages. At present, most studies used this cell to investigate malignant transformation of chemical carcinogens. Furthermore, epithelial tissue-derived tumors account for about 80% of all cancer cases, which gives epithelial cells all advantage over those derived from animal or human fibrocyte. By using human epithelial cells, researches on tumor genesis and development could ideally imitate the tumors natural progress in human.

Previous studies showed that it was different to cause malignant transformation when attack epithelial cells by GMA once only[9-10]. So in this study, we used the GMA at the dose of 1-32 μg/mL to induce 16HBE malignant transformation. The result of cytotoxicity test showed that the cell viability decreased with the increase of exposure dose. For transformed cells losing contact inhibition, we scanned transformed cells with electron microscope to observe the rough surface of cells and found more microvilli which were thick and with varied length, and the pseudopodia extending around to form a malignant phenotype.

In this study, we used the armpit and back of nude mice to inoculate the transformed cells, the tumor formation and development in nude mice could be observed obviously at the second week after implantated. The histopathology test showed that the tumors were the squamous cell carcinoma which strongly suggested the malignant characteristics of transformed cells.

In this study we used the chip of 'NimbleGen HG18 CpG Promoter Microarray Methlation' which was the only one CpG promoter region covering chip[11-13]. The results of 16HBE malignant transformation at different stages induced by GMA in our study would be the basis for the screening of malignant transformation cells at different stages of methylated genes. The occurrence of gene methylation is orderly and the strictly controlled in the development process[14-15]; methylated genes (including the degree of methylation) will vary with the development process.

We observed dynamic changes of genes (Such as tumor suppressor genes, repair genes) methylation in 16HBE malignant transformation process induced by GMA, and different methylation status of genes at different stages could provide a clue for analysis of exposure of GMA[16-18]. Based on the above analy sis, at different stages of transformation, the occurence

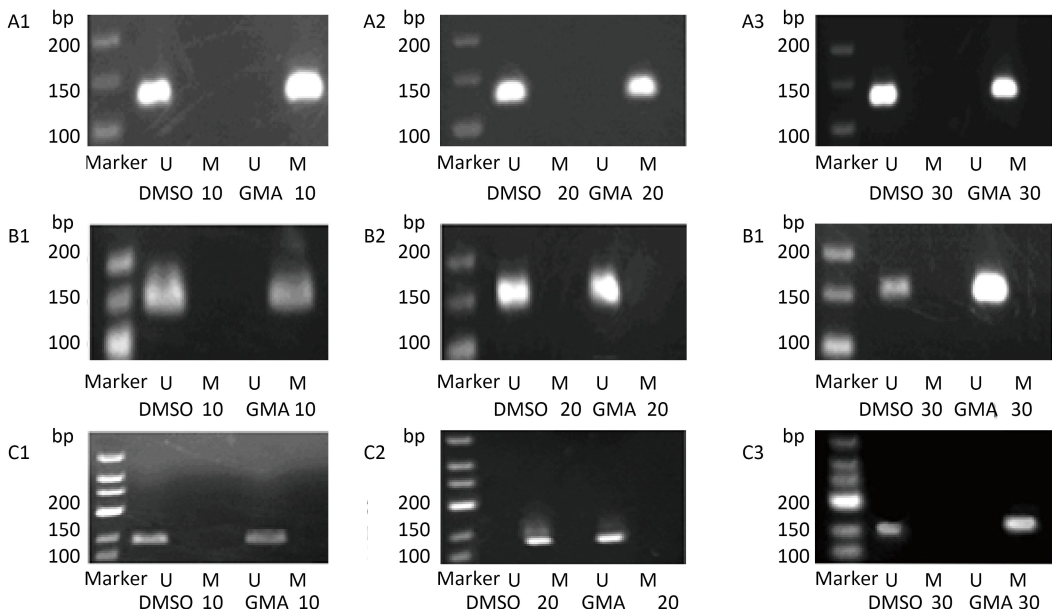


Figure 6. Methylation status of genes at different stages of 16HBE cells malignant transformation induced by GMA. DMSO: Same stage of DMSO; GMA: Cell of GMA; U: non-methylation-specific; M: methylation-specific; A: *OPCML*; B: *THBS1*; C: *P15*.

of methylation was in different genes, and the methylation status of single genes will change in the process of transformation.

Considering the existing research methods, we chose the fast MSP method to verify DNA methylation status by screening analysis[19-20]. *OPCML* is widespread in normal tissues and ovarian epithelial tissue, and plays an important role as an adhesion factor in maintaining normal cell function. *OPCML*[21] and LSAMP (LAMP), NEGRl (KILOM) and HNT (Neurot rimin), reported in the literature, constitute lgLONs subfamily belong to immunoglobulin superfamily belonging to immunoglobulin superfamily which is a cell surface adhesion molecules. By analysis of structure and biological function, we found that the approximate sequence of tyrosine kinase growth factor receptor by intracellular second messengers may be the other way to make gene lose activity and lead to the formation of tumor; the IgLON family act on cell adhesion as well as mutual recognition, and thus the gene may be the same as the other members of the family playing a negative regulatory role in tumor cell invasion and metastasis. The results of MSP showed that the gene of *OPCML* methylation occurred at every stage in the malignant transformation. which verified the results of chip. In the process of malignant transformation, we saw changes not only in biological characteristics but also at the molecular level, which reflected the malignant transformation of 16HBE cells caused by GMA.

THBS1 (Thrombospondin-1, *THBS1*, thrombospondin-1) gene located on chromosome 15q15, and its encoded protein is a matrix adhesion glycoprotein, which can be synthesized by multi- kinds of human tissues including platelets, endothelial cells into fibroblasts, smooth muscle cells. *THBS1* found in platelet a granule composition is the strongest angiogenic negative regulator which can regulate cell adhesion, migration, proliferation and differentiation-induced platelet aggregation and inhibit vascular generation[22]. Recent studies showed that the *THBS1* was involved in the occurrence of malignant tumors, and its expression levels and tumor progression were negatively correlated. In this study, consistent with the results of chip, the MSP showed that the gene of *THBS1* methylation occurred in protophase but not in metaphase and anaphase. So the *THBS1* gene can be regarded as an early sensitive indicator of GMA to 16HBE malignant transformation[22-23].

P15 gene is a cyclin kinase inhibitory factor in family members. Gene methylation leads to disorders of the cell cycle, promotes cell dysplasia and cancer. Studies have found that the gene remaining at the G0/G1 phase is a typical tumor suppressor gene. Its 5'-CpG methylation and non-Hodgkin's malignant showed a high degree of positive correlation between the degree of malignancy which suggested that the gene mutation is a index of non-Hodgkin's disease[24-26]. The results of this study showed that the gene of *P15* occurred methylation both in metaphase and anaphase but not in protophase. Which were consistent with the chip. The higher degree of malignancy, the more opportunity to methylation. So it suggested that *P15* gene methylation can be used as a special kind of gene mutation index in the malignant transformation process, and may be one of the reasons for the cell model of malignant transformation.

In this study, three genes were selected from microarray results. We use the MSP to assay DNA methylation status at the different stages of the process of 16HBE malignant transformation induced by GMA, the detection results were consistent with the microarray results which inferred that these genes might be considered as the characteristics of genes of different conversion stage, and provide clues for the screening of the GMA early tumor biological indicators[16] .

In summary, according to the results of methylation chip, we found that the pattern of DNA methylation could change in the process of 16HBE malignant transformation induced by GMA. Then we chose some genes to detect their DNA methylation patterns, and found that it was the same with previous result. The results showed that abnormal methylation in CpG island of *OPCML* can be considered it as an specific genes occured in the process of 16HBE malignant transformation by GMA; *THBS1* can be considered as an early sensitive index; *P15* can be considered as an cell malignant relative biological index of 16HBE malignant transformation by GMA. It provides new clues on carcinogenicity risk evaluation of GMA, epidemiologic monitoring and mechanism study on its chemical carcinogenesis.

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