

Genes Differentially Expressed in Human Lung Fibroblast Cells Transformed by Glycidyl Methacrylate¹

XUE-JUN YIN^{*,#3}, JIAN-NING XU[#], CHANG-QI ZOU[#], FENG-SHENG HE[#], AND FU-DE FANG^{*,2}

^{*}*Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing 100005, China;*

[#]*Institute of Occupational Health and Poisoning Control, Chinese Center for Disease Control and Prevention, Beijing 100050, China*

Objective To define the differences in gene expression patterns between glycidyl methacrylate (GMA)-transformed human lung fibroblast cells (2BS cells) and controls. **Methods** The mRNA differential display polymerase chain reaction (DD-PCR) technique was used. cDNAs were synthesized by reverse transcription and amplified by PCR using 30 primer combinations. After being screened by dot blot analysis, differentially expressed cDNAs were cloned, sequenced and confirmed by Northern blot analysis. **Results** Eighteen differentially expressed cDNAs were cloned and sequenced, of which 17 were highly homologous to known genes (homology = 89%-100%) and one was an unknown gene. Northern blot analysis confirmed that eight genes encoding human zinc finger protein 217 (ZNF217), mixed-lineage kinase 3 (MLK-3), ribosomal protein (RP) L15, RPL41, RPS16, TBX3, stanniocalcin 2 (STC2) and mouse ubiquitin conjugating enzyme (UBC), respectively, were up-regulated, and three genes including human transforming growth factor β inducible gene (Betaig-h3), α -1,2-mannosidase 1A2 (MAN 1A2) gene and an unknown gene were down-regulated in the GMA-transformed cells. **Conclusion** Analysis of the potential function of these genes suggest that they may be possibly linked to a variety of cellular processes such as transcription, signal transduction, protein synthesis and growth, and that their differential expression could contribute to the GMA-induced neoplastic transformation.

Key words: Glycidyl methacrylate; Neoplastic transformation; mRNA differential display; Transformation-related genes

INTRODUCTION

Glycidyl methacrylate (GMA) is an important chemical widely used in resin, coating, adhesive and plastic industries. It has been reported that GMA displayed evident genotoxic and epigenotoxic effects in various *in vivo* and *in vitro* test systems, including primary DNA damage^[1-3], chromosomal damage^[1,4], and gene mutations^[3,4]. Our recent *in vitro* studies showed that GMA induced morphological transformation of several types of mammalian or human cells with neoplastic properties, demonstrating its carcinogenic potential^[3,5-7]. While

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²Correspondence should be addressed to Fu-De FANG. Tel: 86-10-65105092. Fax: 86-10-65253005. E-mail: fangfd@public3.bta.net.cn

³Current address: School of Pharmacy, West Virginia University, P. O. Box 9530, Morgantown, WV26506, USA. Tel: (304)-293-1694. Fax: (304)-293-2576. E-mail: xyin@hsc.wvu.edu

Biographical note of the first author: Xue-Jun YIN, male, born in 1963, M. D., Ph. D., associate professor, majoring in environmental and occupational toxicology.

mutations and abnormal expression of p53, c-myc and GST-*p* have been suggested to play important roles in GMA-induced transformation^[5-7], there has been limited characterization of the global changes in expression of genes and their relationship with the activation of specific signal pathways or molecular/cellular events during this process. Up to the present, the molecular mechanism by which GMA induces cell transformation and the process of neoplastic changes in the transformed cells remain largely unknown.

Central to any attempt to understand the complex molecular basis for transformation is defining the differences in gene expression profiles between related normal and neoplastic cell populations. Since *in vitro* cell transformation is highly relevant to the process of *in vivo* carcinogenesis and considered a useful model for studies of tumor induction and neoplastic growth, knowledge of malignant cell transformation, particularly in human cells, should facilitate our understanding of carcinogenesis in human beings. It is considered that malignant transformation arises from the accumulation of gene mutation and/or abnormal expression within the target cells. The application of mRNA differential display polymerase chain reaction (DD-PCR) technique allows rapid and sensitive comparison of mRNA expression in related cell populations without prior hypothesis as to which genes should be examined and so increases the possibility of identifying completely novel and unexpected changes in certain cellular process. The basic strategy for DD-PCR is a reverse transcription of the cellular mRNA pool followed by an amplification using a set of anchored oligo (dT) primers and of arbitrary decamers^[8]. To explore the molecular basis underlying the transformation process induced by GMA, we used the DD-PCR technique to screen for genes expressed differentially between GMA-transformed human lung fibroblast cells and controls. The purpose of this study is to identify genes whose expression is altered in association with neoplastic transformation induced by GMA. Our findings revealed that the expression levels of those genes whose function was involved in transcription, signal transduction, protein synthesis and growth were modulated during the transformation occurring in the cells.

MATERIAL AND METHODS

Cells and Cell Culture

The transformed cells and controls were derived from cultures of 2BS human lung fibroblast cells (2BS cells, Beijing Research Institute of Biological Products, Beijing, China) in an *in vitro* transformation assay as described elsewhere^[3]. Briefly, 2BS cells were treated three times with 8 µg/mL of GMA or dimethyl sulphoxide (DMSO, 0.1%) for 72 h with a 24 h interval after each treatment. The cells were subsequently fed twice weekly with fresh Eagle's minimum essential medium (MEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 4 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated (56°C for 30 min) fetal bovine serum and maintained at 37°C in a humidified atmosphere containing 5% CO₂ for an additional 10 weeks. At the end of incubation period, the transformed cells were isolated from type III foci in GMA-treated flasks and subcultured three times to obtain a sufficient number for RNA isolation. Cells derived from DMSO-treated cultures, subcultured similarly, served as controls. The transformed cells, other than controls, exhibited various malignant transformation phenotypes such as evident morphological changes, anchorage-independent growth, increased agglutination toward agglutinin, karyotypic alterations and chromosomal aberrations.

Differential Display

Differential display was performed based upon the method of Liang and Pardee^[8]. Total RNA was isolated from cultured transformed and control cells when 80%-90% confluent on 75 cm² tissue culture flasks using Trizol reagent (Gibco BRL). RNase-free DNase I-treated RNA (2 µg) from each cell population was used for mRNA reverse transcription with a SuperScript Preamplification System Kit (Gibco BRL), according to the manufacturer's instructions. Briefly, reverse transcription of mRNA was performed with each of three 3' anchor primers (T₁₁A: 5'-CGCGGATCCTTTTTTTTTTTTA-3', T₁₁C: 5'-CGCGGATCCTTTTTTTTTTTTC-3' and T₁₁G: 5'-CGCGGATCCTTTTTTTTTTTTG-3', Shanghai Sangon Ltd., Shanghai, China), in a 20 µL reaction containing 2.5 mmol/L MgCl₂, 0.01 µmol/L DTT, 0.5 mmol/L dNTPs, and 200 U SuperScript II reverse transcriptase at 42°C for 50 min. The resulting cDNAs were then amplified by PCR in 10 µL of reaction mixture containing 2 µL of reverse-transcribed products, 1×PCR buffer, 2.5 mmol/L MgCl₂, 2.5 µmol/L dNTPs, 0.2 µmol/L 3' anchor primers, 0.2 µmol/L 5' arbitrary primers

(AP₁: 5'-CGGAATTCTCCAGTTCAG-3', AP₂: 5'-CGGAATTCCAAGCGAGGT-3', AP₃: 5'-CGGAATTCCTTGTAGCCA-3', AP₄: 5'-CGGAATTCCTTTCTAGCC-3', AP₅: 5'-CGGAATTCACCTTGGACA-3', AP₆: 5'-CGGAATTCATGCTGGGGA-3', AP₇: 5'-CGGAATTCATGC TGGTAG-3', AP₈: 5'-CGGAATTCATGCTGTATG-3', AP₉: 5'-CGGAATTCTCGGTCATAG-3', and AP₁₀: 5'-CGGAATTCGATCTGACTG-3', Shanghai Sangon Ltd.), 0.25 µL of α-[³²P]dCTP (3.7×10⁹ Bq/L, Beijing Yuhui Co., Beijing, China), and 0.5 unit of Taq Plus polymerase (Shanghai Sangon Ltd.). The optimal reaction conditions were as follows: 5 min of incubation at 94°C; 2 cycles of incubation at 94°C for 30 s, at 40°C for 40 s and at 72°C for 5 min; and 30 cycles of incubation at 94°C for 30 s, 52°C for 40 s, and at 72°C for 2 min, followed by a 5-min incubation at 72°C. A portion (3.5 µL) of the radiolabeled PCR products was then electrophoresed on a 6% polyacrylamide gel. Differences in the banding patterns between the two cell populations were revealed by autoradiography of the dried gel. Bands identified and recovered from the sequencing gel were reamplified with the original primer set and run on 1.2% agarose gel.

Dot Blot Analysis

To reduce the workload on the following cloning and sequencing of the cDNA fragments, we performed a dot blot analysis to screen these differentially expressed cDNA for their relative expression levels in the two cell populations. Reamplified cDNAs (400 ng) were spotted onto nylon membranes (GeneScreen plus, DuPont) by using a vacuum blotter (Bio-Rad, Hercules, CA, USA), and blots were baked at 80°C under a vacuum pressure of -25 mm Hg for 2 h. Prehybridization was carried out in a prehybridization/ hybridization solution (6×SSC, 5×Denhardt's solution, 1% SDS, 50% formamide, and 100 µg/mL heat-denatured salmon perm DNA) at 42°C for 1 h. The blots were subsequently hybridized overnight at 42°C with α-[³²P]dCTP-labelled complex cDNA probes generated from reverse transcription of the total RNAs from the transformed cells and controls with the mixed oligo(dT) primers (mixture of T₁₁A, T₁₁C and T₁₁G in equal moles), and washed sequentially with a buffer containing 2×SSC and 1% SDS (washing buffer I) at room temperature for 20 min and with a buffer containing 0.2×SSC and 0.1% SDS (washing buffer II) at 50°C for 40 min, followed by autoradiography. The relative expression levels of each tested gene fragment in the two cell populations were determined by measuring the density of the corresponding hybridization spots with an image analysis system (Image-Pro Plus, Media

Cybernetics, Inc., Silver Spring, MD, USA). This procedure allows us to fast screen hundreds of differentially expressed cDNA and compare their relative expression levels at the same time.

Cloning and Sequencing of cDNA Fragments

Eighteen cDNA fragments that showed highly differential expression in dot blot analysis were selected to be cloned. The cDNA fragments were reamplified by PCR, then recovered from the gel by excision and purified with a gel extraction kit (Qiagen Inc., Chatsworth, CA, USA). cDNA clone was carried out using a TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA), 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. Plasmid DNA was isolated from overnight cultures of the positive colonies using a Qiaprep Spin Miniprep Kit (Qiagen Inc.). Colonies containing plasmids with inserts were identified and the approximate sizes of the cDNA inserts were determined by excision with EcoRI restriction enzyme (Promega Co., Madison, WI, USA), followed by electrophoresis on 1.2% agarose gel. Sequencing of the miniprep DNA was performed with either M13 reverse primer or T7 primer using a Dye Terminator ABI PRISM kit (Perkin Elmer, Foster City, CA, USA) and analyzed on an ABI 373 DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA). Alignment of insert sequences obtained was made by using the Chromas program (version 1.43, Griffith University, Brisbane, Queensland, Australia) and the BLASTN and FASTA programs were used to search for homologous sequences in GenBank/EMBL databases.

Northern Blot Analysis

Northern blotting was performed by using the standard methods. Briefly, total RNA (15 µg) extracted from the same cell populations as described in the previous section, was denatured and separated on a 1.2% agarose-formaldehyde gel by electrophoresis. The RNA was transferred onto nylon membranes (GeneScreen, DuPont) by standard capillary blotting techniques. Blots were then baked at 80°C for 2 h, prehybridized in the prehybridization/hybridization solution at 42°C for 1 h, and hybridized with α -[³²P]dCTP random primer-labelled cDNA probes (Prime-a-Gene Labelling System kit, Promega Co.) overnight at 42°C. After hybridization, the blots were washed sequentially with the washing buffer I at room temperature for 20 min and with the washing buffer II at 55°C for 40 min, followed by autoradiography. Each blot was then reprobed with labelled β -actin cDNA to confirm equal loading.

RESULTS

Comparison of mRNA Expression in Transformed and Control Cells by Differential Display

To identify transcriptionally regulated genes potentially involved in GMA-induced cell transformation, we compared mRNA differential display patterns for the GMA-transformed cells and controls. Significant differential gene expression between the two cell populations was observed (Fig. 1). Two hundred twenty-six differentially expressed cDNA fragments were initially recognized and recovered from the sequencing gels, of which 222 were then successfully reamplified with PCR. After further screening by dot blotting, 101 differentially expressed cDNA fragments including 57 up-regulated and 44 down-regulated in the

transformed cells were demonstrated (data not shown).

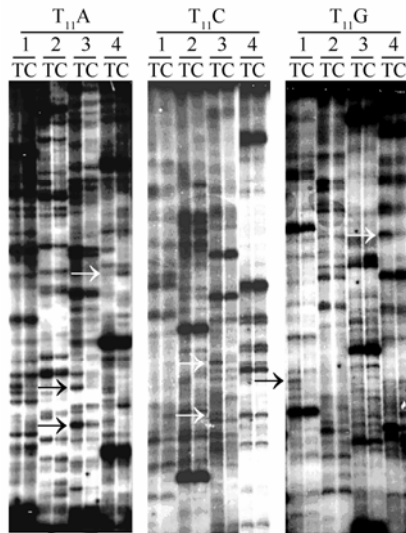


FIG. 1. mRNA differential display comparison of GMA-transformed 2BS cells and controls. cDNAs were synthesized by reverse transcription of total RNA isolated from the transformed (T) and control (C) cells, amplified by PCR and resolved on a 6% polyacrylamide sequencing gel. Primers used in DD-PCR are listed on top of each panel. 1. AP₁, 2. AP₂, 3. AP₃, 4. AP₄. Arrowheads, examples of bands corresponding to the differentially expressed genes.

Cloning, Sequencing and Homology Analysis

Eighteen cDNA fragments chosen from those displayed highly differential expression in dot blot analysis were cloned and sequenced. Of the 18 sequence homology searches performed 17 were highly homologous or nearly identical to known genes (homology=89%-100%, Table 1). To date we found no significant homology with any published gene for the sequence obtained from clone YG71, suggesting that it represents previously unknown gene, which may be associated with GMA-induced cell transformation.

Northern Blot Analysis

All cloned cDNA fragments were analyzed by Northern hybridization of which 11 were finally confirmed to be differentially expressed (Table 1, Fig. 2). Eight cDNA fragments designated as ZA25, ZG61, ZA21, ZC91, ZG74, ZA11, ZG21, and ZC103, which represent human zinc finger protein 217 (ZNF217), mixed-lineage kinase 3 (MLK-3), ribosomal protein (RP) L15, RPL41, RPS16, TBX3, stanniocalcin 2 (STC2) and mouse ubiquitin conjugating enzyme (UBC), respectively, showed higher levels of expression in the transformed cells than that in controls. The other three cDNA fragments, designated as YG72, YC22, and YG71, which represent human transforming growth factor β (TGF- β) inducible gene (Betaig-h3), α -1,2-mannosidase 1A2 (MAN 1A2) gene and an unknown gene, respectively, were down-regulated in the transformed cells. The expression pattern of the remaining seven cDNA fragments, however, was not reproducibly confirmed by Northern blotting, while their differential expression was shown in both differential display and the dot blot analysis. Five of these fragments (YG73, YG101, ZA22, ZG26, and ZG51)

TABLE 1

Homologous Analyses of cDNA Differentially Expressed in GMA-Transformed 2BS Cells				
cDNA	Fragment Size (bp)	GenBank/EMBL Match	Homology (%)	Expression Profile ^a
ZA11	383	Human TBX3	98	A
ZA21	515	Human ribosomal protein L15 (RPL15)	97	A
ZA25	355	Human zinc finger protein 217 (ZNF217)	97	A
ZC91	418	Human ribosomal protein L41 (RPL41)	99	A
ZC103	320	Mouse ubiquitin conjugating enzyme (UBC)	89	A
ZG21	659	Human stanniocalcin 2 (STC2)	96	A
ZG61	251	Human mixed lineage kinase (MLK-3)	100	A
ZG74	149	Human ribosomal protein S16 (RPS16)	100	A
YC22	443	Human α -1,2-mannosidase 1A2 (MAN1A2)	99	B
YG71	562	Novel gene ^b	-	B
YG72	478	Human TGF- β inducible gene (Betaig-h3)	98	B
ZA22	499	Human ferritin H chain	96	C
ZG26	213	Human cyclophilin B	100	C
ZG51	459	Human G protein α i subunit	98	C
YG73	343	Human DNA sequence from clone RP5-1046G13	99	C
YG101	766	Human H2A histone family, member Z	98	C
ZC43	202	Human polycystic kidney disease type II protein	96	D
YG91	357	Human cDNA clone DKFZp434M1616	97	D

Note. ^aConfirmed by Northern blot analysis as indicated in Fig. 2. A, up-regulated in the transformed cells; B, down-regulated in the transformed cells; C, no differential expression found; D, no signal detected. ^bGenBank accession no.: AW600317.

showed similar expression levels between the transformed cells and controls, and the signals in both cell populations for the two others (ZC43 and YG91) were not detected (data not shown). Occurrence of these non-differentially expressed fragments is most likely due to the artifactual isolation of the false-positive clones, a major inherent drawback of the DD-PCR technique as discussed previously by many investigators^[9].

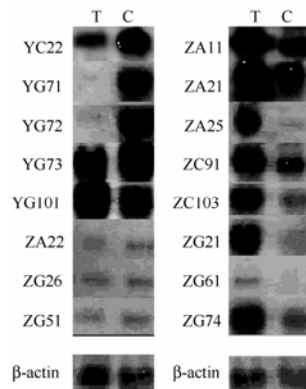


FIG. 2. Northern blot analysis of mRNA expression in GMA-transformed 2BS Cells and controls. Total RNA (15 μ g) isolated from the transformed cells (T) and controls (C) was loaded into each lane and hybridized with [³²P]dCTP-labeled cDNAs obtained from cloned fragments after the differential display analysis. Each blot was then probed with labeled β -actin cDNA to confirm equal loading.

DISCUSSION

A key interest of this study is to identify genes potentially involved in GMA-induced cell transformation. Clues provided by the biological functions of the known genes defined within the context of the present experiment indicate that genes that play regulatory roles in particular processes such as transcription, signal transduction, protein synthesis and growth are responsive to GMA exposure. Although the causal relationship between the expression of these genes and the expression of the neoplastic phenotype has not yet been evaluated, it is of interest to note that most of the known genes identified have been previously shown to be correlated with the expression of the neoplastic phenotype. For example, TBX3, a member of the T-box family of transcription factors, is capable of immortalizing mouse embryo fibroblasts and transforming these cells together with *myc* or oncogenic *ras* by suppressing the induction of tumor suppressor p14ARF, which is thought to fulfill an important protective role in preventing primary cells from oncogenic transformation *via* its action in the p53 pathway^[10,11]. ZNF217, a human breast cancer transcription factor gene recently cloned from breast cancer cells, is considered a good candidate for oncogene^[12]. DNA sequence analysis suggests that ZNF217 encodes alternately spliced, Kruppel-like transcription factors which have been implicated in several human malignancies such as leukemia, glioblastoma, Wilms tumor, gastric carcinoma and breast cancer. Nonet *et al.*^[13] recently showed that overexpression of ZNF217 gave rise to immortalization in finite life span human mammary epithelial cells. Our identification of TBX3 and ZNF217 genes in the GMA-transformed cells suggests that aberrant expression of these tumor-related genes may enable the 2BS cells to lose tumor-suppressor function or overcome senescence, allowing them to continue growing and accumulating other changes necessary for malignant progression. These results further support the hypothesis that GMA induces cell transformation by altering expression and/or function of some cellular protooncogenes, and validates effectiveness of the DD-PCR technique and reasonableness of our experimental model.

MLK-3 is a widely expressed serine/threonine kinase that bears multiple protein interaction domains. Although the precise function of MLK-3 is unknown, studies have indicated that it functions as an upstream activator in regulating signaling events of c-Jun-terminal kinases (JNKs), and to a lesser extent, p38 mitogen-activated protein kinases (MAPKs) pathways^[14]. Though p38 MAPK appears to be involved in apoptosis, JNKs have been shown to play a major role in promoting cell transformation by both oncogene (e.g., *ras*) and growth factor-mediated pathways, mainly through the activation of downstream transcription factor c-Jun^[15]. Thus, the aberrant expression and/or functioning of MLK-3, through the activation of JNKs pathway, might have significant effect on cell transformation as well as other cellular processes. Interestingly, the gene encoding MLK-3 has been mapped to 11q13.1-q13.3^[16], a chromosomal region linked with the development of a number of human malignancies, raising the possibility that MLK-3 may play a role in oncogenesis. The up-regulation of MLK-3 in GMA-transformed cells in the present study suggests that this gene may be involved in GMA-induced transformation, and the activation of JNKs pathway may play a role in this process.

Betaig-h3 was first identified as a TGF- β responsive gene in a human lung adenocarcinoma cell line^[17]. Since it was induced in several cell lines whose proliferation is affected by TGF- β , Betaig-h3 may be involved in mediating some of the signals of TGF- β , which is believed to play a bifunctional role during the process of carcinogenesis, retarding carcinoma development but enhancing progression once neoplastic transformation has occurred and the growth inhibitory response to TGF- β has been lost^[18]. Recently, a decrease in expression of

Betaig-h3 has been noted in transformed cells and tumors, leading to the hypothesis that Betaig-h3 may function as a tumor suppressor gene. Zhao *et al.*^[19,20] demonstrated that the expression of Betaig-h3 was decreased in 14 human tumor cell lines of diverse histological types and in several independently generated, radiation- or asbestos-induced tumorigenic cells, suggesting that loss of Betaig-h3 expression may be a frequent event in human cancer and causally related to acquisition of tumorigenic phenotype in transformed cells. Direct evidence for a tumor suppressor function by Betaig-h3 has also been reported by these investigators who showed ectopic expression of Betaig-h3 in tumorigenic cells resulted in loss of tumorigenic phenotype, inhibition of cell growth *in vitro* and tumorigenicity in nude mice^[20]. In the present study, expression of Betaig-h3 was found to be down-regulated in the GMA-transformed 2BS cells in comparison with controls. We propose that Betaig-h3 expression may be important for regulation of proliferation in 2BS cells, probably serving as a protein module bridging TGF- β with downstream signal pathway components. Since the loss of responsiveness of different tumor cells to the antiproliferative effects of TGF- β is a common feature in carcinogenesis, it will be interesting to determine if the expression of Betaig-h3 in the transformed cells will affect their neoplastic phenotype and responsiveness to the growth inhibitory response to TGF- β .

Homeostasis of ribosomal proteins, the components of the translation machinery, is maintained in normal cells for the controlled production of cellular proteins. Augmentation in the capacity of the translation apparatus is a prerequisite for cell proliferation driven by either oncogenes or mitogenic stimulation. An increasing number of ribosomal proteins has been reported to have relationship with malignant progression, transformation and tumor formation. Specially, RPL41, which was shown to be up-regulated in fos- and src-transfected rat fibroblasts and in a series of human malignant tumors and cell lines, can enhance the autophosphorylation of protein kinase CKII which plays a critical role in cell growth, proliferation and tumorigenesis^[21]. RPS16, first isolated from Panc 1 human pancreatic tumor cells, was shown a 30-fold higher expression in this poorly differentiated tumor cell line than its well differentiated counterpart^[22]. Excessive expression of RPL15 which has been suggested to play a role in carcinogenesis of esophagus, was also found in human hepatocellular carcinoma and esophageal cancer^[23,24]. Our finding that RPL15, RPL41 and RPS16 were up-regulated in GMA-transformed 2BS cells supports the notion that overexpression of certain types of ribosomal proteins may be the consequence of transformation, or alternatively, expression of these ribosomal proteins may be associated with functions related to growth or survival in the transformed cells. These results also suggest that the accumulation of ribosomal subunits and the rate of protein synthesis may be important modulators of GMA-induced cell growth and transformation.

Ubiquitin-mediated degradation of regulatory proteins plays important roles in the control of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis^[25]. Abnormalities in ubiquitin-mediated processes have been shown to cause pathological conditions, including malignant transformation. It has been reported that HR6B, a human UBC, was found to be up regulated in metastatic mammary tumor lines and breast carcinomas, as compared with normal or nonmetastatic mammary cells. Furthermore, overexpression of exogenous HR6B in normal human breast epithelial cells induced cell-cell fusion that resulted in generation of multinucleated cells, aneuploidy and ability for anchorage-independent growth^[26]. Our study shows that expression of UBC was up-regulated in the GMA-transformed cells, further suggesting that UBC, through its potential functions in affecting cellular genomic integrity and cell cycle regulation, may play a role in GMA-induced cell transformation. This is consistent with the alterations of genomic stability and mutation/expression of p53 detected

in the GMA-transformed cells^[3,27]. STC2, which encodes a calcium/phosphate homeostatic hormone, has been shown to be overexpressed in estrogen receptor-positive breast cancer cells and strongly induced by 17- β -estradiol, a carcinogenic estrogen which plays a central role in breast cancer development^[28], suggesting its involvement in the development of estrogen-induced breast cancer. However, the role of STC2 in GMA-induced transformation remains unclear. While MAN1A2 is known to play a key role in processing and degradation of cell surface oligosaccharides which have a general influence on growth control^[29], the precise function of it in cell transformation has not yet been reported.

In conclusion, using the DD-PCR technique, we have detected global alterations in the expression profile of genes associated with GMA-induced transformation in an *in vitro* transformation model. The study indicates for the first time that expression of genes with diverse roles in transcription, signal transduction, protein synthesis and growth was deregulated in GMA-transformed 2BS cells, providing evidence that complex mechanisms are involved in this process. At present, however, the basis for the altered expression of genes identified in this study is unclear. The down-regulation of the unknown gene by GMA has also provided a new challenge to understanding of the GMA-induced cell transformation. It should be possible to directly evaluate the function of the candidate genes in GMA-induced multistage transformation models with different neoplastic potential, and such information would help us to better understand the relationship between the expression of a specific gene and development of neoplastic phenotype associated with GMA exposure.

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