MOLECULAR AND CLINICAL ONCOLOGY 2: 1160-1166

Expression of acidosis-dependent genes in human cancer nests

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Received January 24, 2014; Accepted June 30, 2014

DOI: 10.3892/mco.2014.344

Abstract. Previous studies investigating cancer cells cultured at acidic pH have shown that the expression level of ~700 genes were more than two-fold higher than those of the cells cultured in alkaline medium at pH 7.5. The aim of the present study was to confirm whether these acidosis-induced genes are expressed in human cancer tissues. Therefore, 7 genes were selected from our previous study, which encoded interleukin 32 (*IL-32*), lysosomal H^+ transporting ATPase, V0 subunit d2 (*ATP6V0D2*), tumor necrosis factor receptor superfamily, member 9 (*TNFRSF9*), amphiregulin, schwannoma-derived growth factor (*AREG*), v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (*ErbB3*), PRR5-ARHGAP8 (*LOC553158*) and dimethylglycine dehydrogenase (*DMGDH*), and their expression was examined in human clinical specimens from patients with cancer. In addition, the expression of the gene encoding manganese superoxide dismutase (*MnSOD*) was examined. The specimens from patients with colon, stomach and renal cancer showed increased *MnSOD*, *IL-32*, and *TNFRSF9* transcripts compared to those from non-tumorous regions of the same patients. Notably, an elevated expression of *ATP6V0D2* was found in the specimens from patients with stomach cancer, whereas the expression was decreased in those from patients with colon and renal cancer. The expression of *LOC553158* was upregulated in colon and stomach cancer specimens. These results indicate that the investigation of gene expression under acidic conditions is useful for the development of novel cancer markers and/or chemotherapeutic targets.

Introduction

In the central regions of solid tumors, the extracellular pH falls below pH 6.5 as a consequence of lactate accumulation, which is caused by hypoxic conditions produced by a lack of sufficient vascularization (1,2) or an increase in tumor-specific glycolysis combined with impaired mitochondrial oxidative phosphorylation (3). Organ functions may be strongly affected by the disruption of the pH homeostasis as all the organs contain a large number of enzymes with pH-sensitive catalytic activity. Therefore, it can be argued that alternative metabolic processes are activated under acidic conditions to compensate for the decline in processes functioning at alkaline pH.

When various metabolic processes are working under different pH conditions, the efficacy of a number of inhibitors under acidic conditions may be different to those observed in conventional alkaline media. Impaired efficacy of paclitaxel, mitoxantrone and topotecan has been previously reported at pH 6.5 as compared to their efficacy at pH 7.4 in murine EMT6 and human MGH-U1 cells (4), and acidic conditions induced daunorubicin resistance by increasing the activity of p-glycoprotein via p38 activation in rat prostate cancer cells (5).

Malignant pleural mesothelioma is an aggressive tumor associated with asbestos exposure, and its prognosis is extremely poor (6). Mesothelioma shows resistance against numerous chemotherapeutic reagents (7). Our previous study found that statins inhibited the proliferation of mesothelioma cells strongly in an acidic medium with a pH that was close to the pH of an area of cancer *in vivo* (8). Statins, which are inhibitors of mevalonate synthesis, are prescribed for hyperlipidemia as the inhibition of mevalonate synthesis reduces blood cholesterol levels. However, the anti-cancer activity of statins has not been demonstrated *in vitro*. Recently, clinical studies have revealed that stains are effective at attenuating the growth of cancer cells *in vivo* (9,10), in agreement with our previous *in vitro* observations at acidic pH (8). A previous study has shown that the anticancer activity is caused by the inhibition of geranylgeranyl diphosphate, derived from mevalonate, indicated that the function of certain geranylgeranylated proteins is essential for cell proliferation under acidic conditions (8,11). In addition to the investigations with inhibitors, our previous studies found that different signal transduction pathways function under acidic environments (12,13), and that C-Terminus protein of IκB-β, which is an IκB-β variant, acted as a critical transcriptional regulatory factor at pH 6.3 only, and not at pH 7.4 (14,15).

These previous findings indicate that numerous proteins are functioning preferentially under low pH conditions. DNA array analysis showed that the expression of ~700 genes was elevated more than two-fold in mesothelioma cells under acidic conditions compared to in cells cultured in an

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Key words: gene expression, human cancer, acidic environments

alkaline medium (16). Numerous genes were also found to be strongly expressed in breast cancer cells cultured in an acidic medium (17). These gene products may be good candidate therapeutic targets and/or diagnostic markers of cancers. In the present study, the aim was to confirm whether or not the genes with an increased expression in cancer cells cultured in acidic medium are expressed in human cancer nests. A total of 8 genes with an increased expression in mesothelioma cells cultured under acidic conditions were selected and the expression was examined in human specimens from patients with cancer. The expression of the selected genes was demonstrated to be higher in numerous human cancer specimens compared to those in the specimens prepared from the surrounding normal areas.

Materials and methods

Human specimens from patients. Human tumor and the corresponding non-tumorous tissues were obtained from the Chiba Cancer Center Tissue Bank (Chiba, Japan) and used in the study with permission from the Institutional Ethical Committees of Chiba Cancer Center and Chiba University.

RNA extraction from human specimens. The human tissues that were stored at -80°C were mixed with ice-cold TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). After 1 min on ice, the human tissues were homogenized on ice with a homogenizer until the pellets were broken and cell lysis was completed. Total RNA was isolated from the lysate according to the manufacturer's instructions for the TRI reagent.

Quantitative polymerase chain reaction (qPCR). Total RNA (1 μg), prepared as described above, was reverse-transcribed using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) in a total volume of 20 μl containing the random primer for 18S rRNA or the polyT primer for the targeted genes. qPCR amplification was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The PCR reaction was carried out with a mixture containing 12.5 μl PCR Master, 7.5 μM of each sense and antisense primer, 25 ng cDNA, and nuclease-free water in a total volume of 25 μl. The standard thermal profile for PCR amplification was 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The primers used are shown in Table I.

A previous study has reported that the content of ribosomes per cell is ~$4x10^6$ (18), and the amount of mRNA per cell can be estimated using 18S rRNA as a control RNA with the following equation, in which Ct is the threshold cycle number: $4x10^6x2^{\{(Ct\ of\ 18S\ rRNA)\ -\ (Ct\ of\ sample\ RNA)\}}$.

Table I. Primers used in the present study.

Gene name	Sequence
18S rRNA	F: TAGAGTGTTCAAAGCAGGCCC
	R: CCAACAAATAGAACCGCGGT
MnSOD	F: TGA ACG TCA CCG AGG AGA AG
	R: CGT GCT CCC ACA CAT CAA TC
IL-32	F: TCAAAGAGGGCTACCTGGAG
	R: TTTCAAGTAGAGGAGTGAGCTCTG
ATP6V0D2	F: GACCCAGCAAGACTATATCAACC
	R: TGGAGATGAATTTTCAGGTCTTC
TNFRSF9	F: AAACGGGGCAGAAAGAAACT
	R: CTTCTGGAAATCGGCAGCTA
AREG	F: GGGAGTGAGATTTCCCCTGT
	R: AGCCAGGTATTTGTGGTTCG
ErbB3	F: TGCAGTGGATTCGAGAAGTG
	R: GGCAAACTTCCCATCGTAGA
LOC553158	F: AGCCTCCCAGAGCACAACTA
	R: ATGGCCAGATCAAATTCAGC
DMGDH	F: GAGCTCACGGCTGGATCTAC
	R: CCACCACCTGACCAGTTTCT

MnSOD, manganese superoxide dismutase; *IL-32*, interleukin 32; *ATP6V0D2*, lysosomal H^+ transporting ATPase, V0 subunit d2; *TNFRSF9*, tumor necrosis factor receptor superfamily, member 9; *AREG*, amphiregulin, schwannoma-derived growth factor; *ErbB3*,v-erb-b2 erythroblastic leukemia viral oncogene homolog 3; *LOC553158*, PRR5-ARHGAP8; *DMGDH*, dimethylglycine dehydrogenase.

Results

Quantification of mRNA levels in human cancer specimens. Our previous study showed that the expression of 58 genes was elevated more than three-fold in mesothelioma cells cultured for 24 h in an acidic medium (16). The 58 genes are listed in Table II. Seven genes were selected of the 58 genes with various functions, which were interleukin 32 (*IL-32*), lysosomal H^+ transporting ATPase, V0 subunit d2 (*ATP6V0D2*), tumor necrosis factor receptor superfamily, member 9 (*TNFRSF9*), amphiregulin, schwannoma-derived growth factor (*AREG*), v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (*ErbB3*), PRR5-ARHGAP8 (*LOC553158*) and dimethylglycine dehydrogenase (*DMGDH*), and the expression of these genes was examined in human cancer specimens. In addition, the expression of the gene encoding manganese superoxide dismutase (*MnSOD*) was examined as MnSOD has been reported to participate in gastric and colorectal tumor metastasis (19,20), although the expression of *MnSOD* at acidic pH was 1.6-fold in mesothelioma cells. The selected genes are shown in Table II.

One problem in the measurement of mRNA using qPCR was determining which was useful as a control RNA. Thus far, a reference gene, such as *GAPDH*, has generally been used in studies. There are no previous data to show that the expression of such reference genes is stable at acidic pH, particularly in human cancer nests. The amount of 18S rRNA was constant in mesothelioma cells at acidic and alkaline pH (data not shown). The amount of 18S rRNA in total RNA isolated from human cancer specimens was measured, with the results demonstrating that the content of 18S rRNA was constant in all the cancer specimens (Table III). The amount of 18S rRNA was slightly higher in normal areas, but the difference was <2-fold. These data indicated that 18S rRNA was suitable for use as

Table II. Genes with an elevated expression of >3-fold at acidic pH.

Gene	Expression at pH 6.7 (fold)[a]	Relative amount[b]	Description
RHCE	7.816	0.58	Rh blood group, CcEe antigens
RSPO3	7.346	0.70	R-spondin 3 homolog (*Xenopus laevis*)
ZSCAN4	6.346	1.06	zinc finger and SCAN domain containing 4
ErbB3[c]	5.997	0.69	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
AREG[c]	5.650	0.92	amphiregulin (schwannoma-derived growth factor)
FLJ33706	5.579	1.75	hypothetical protein FLJ33706
TNFRSF9[c]	5.464	2.58	tumor necrosis factor receptor superfamily, member 9
BMP1	5.186	0.40	bone morphogenetic protein 1
PIPOX	5.069	0.66	pipecolic acid oxidase
LOC653193	4.485	0.43	similar to Amphiregulin precursor (AR) (Colorectum cell-derived growth factor) (CRDGF)
DMGDH[c]	4.310	0.39	dimethylglycine dehydrogenase
LOC553158[c]	4.306	0.44	PRR5-ARHGAP8 fusion
KCTD19	4.231	0.33	potassium channel tetramerisation domain containing 19
ZC3H6	4.220	0.15	zinc finger CCCH-type containing 6
SIGLEC1	4.184	0.29	sialic acid binding Ig-like lectin 1, sialoadhesin
GRHL3	4.142	0.54	grainyhead-like 3 (Drosophila)
FBXO32	4.117	1.49	F-box protein 32
BMP2	4.014	0.48	bone morphogenetic protein 2
LXN	3.987	9.88	latexin
INPP5D	3.967	0.49	inositol polyphosphate-5-phosphatase, 145kDa
RARRES1	3.882	0.49	retinoic acid receptor responder (tazarotene induced) 1
NYD-SP14	3.847	0.48	NYD-SP14 protein
RRAD	3.827	4.50	Ras-related associated with diabetes
VWCE	3.790	2.35	von Willebrand factor C and EGF domains
ATP6V0D2[c]	3.778	0.69	ATPase, H^+ transporting, lysosomal 38kDa, V0 subunit d2
CDH15	3.750	0.64	cadherin 15, M-cadherin (myotubule)
HES2	3.723	0.54	hairy and enhancer of split 2 (Drosophila)
IL-32[c]	3.711	8.91	interleukin 32
CRELD1	3.707	2.92	cysteine-rich with EGF-like domains 1
PPP1R3E	3.702	0.39	protein phosphatase 1, regulatory (inhibitor) subunit 3E
CLDN14	3.560	0.20	claudin 14
ARHGAP8	3.547	0.23	Rho GTPase activating protein 8
MGC33926	3.508	5.58	hypothetical protein MGC33926
LOC390937	3.497	0.34	similar to ETS domain transcription factor ERF
FUT5	3.486	0.41	fucosyltransferase 5 (α (1,3) fucosyltransferase)
CLEC4F	3.459	0.47	C-type lectin domain family 4, member F
LOC644893	3.363	0.21	hypothetical protein LOC644893
C11orf34	3.359	0.83	chromosome 11 open reading frame 34
EGR4	3.353	0.13	early growth response 4
FLJ42258	3.324	0.56	FLJ42258 protein
CFB	3.320	5.25	complement factor B
GPR78	3.302	0.92	G protein-coupled receptor 78
MUC3B	3.300	0.49	mucin 3B, cell surface associated
CRYM	3.298	1.48	crystallin, μ
CYYR1	3.294	0.14	cysteine/tyrosine-rich 1
LOC196394	3.286	7.17	hypothetical protein LOC196394
LOC644725	3.262	0.30	similar to γ-tubulin complex component 3 (GCP-3) (Spindle pole body protein Spc98 homolog) (hSpc98) (hGCP3) (h104p)

[a]Expression ratio in cells cultured at pH 6.7 for 24 h compared to pH 7.5; [b]percent ratio of the mRNA level to the level of 18S rRNA at pH 6.7; [c]selected genes.

Table II. Continued.

Gene	Expression at pH 6.7 (fold)[a]	Relative amount[b]	Description
FGF7	3.219	0.17	fibroblast growth factor 7 (keratinocyte growth factor)
PNLIPRP3	3.178	1.21	pancreatic lipase-related protein 3
C1orf101	3.170	0.13	chromosome 1 open reading frame 101
ALS2CR7	3.164	0.49	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 7
IGLL1	3.130	1.12	immunoglobulin λ-like polypeptide 1
GDF15	3.112	22.10	growth differentiation factor 15
FLJ26850	3.082	0.23	FLJ26850 protein
PTP4A3	3.037	9.05	protein tyrosine phosphatase type IVA, member 3
TAS2R39	3.035	0.34	taste receptor, type 2, member 39
SGK2	3.015	0.28	serum/glucocorticoid regulated kinase 2
CRNN	3.005	0.20	cornulin
MnSOD[c]	1.599	13.77	manganese superoxide dismutase
GAPDH	0.962	100.00	glyceraldehyde-3-phosphate dehydrogenase

[a]Expression ratio in cells cultured at pH 6.7 for 24 h compared to pH 7.5; [b]percent ratio of the mRNA level to the level of 18S rRNA at pH 6.7, [c]selected genes. For original DNA array data, see reference 16.

Table III. Amount of 18S rRNA in the human specimens from patients with colon, stomach, liver and renal cancer.

Tissues	Samples, n	Ct of 18S rRNA (mean ± SD)	
		Normal area	Cancer area
Colon	11	11.07±0.64	11.71±0.58
Stomach	10	11.03±0.55	11.49±1.01
Renal	10	10.97±0.69	11.60±0.40
Liver	10	10.63±0.54	11.63±0.66
Total	41	10.93±0.61	11.61±0.67

SD, standard deviation.

control RNA. The Ct value shown in Table III was similar to that observed in cells cultured *in vitro* (data not shown), suggesting that the ribosome content per cell is constant even when the activity of protein synthesis varies. As one cell was reported to have ~$4x10^6$ ribosomes (18), the approximate copy number of mRNA can be calculated using this number. The mRNA level of *GAPDH* estimated using 18S rRNA as a control RNA decreased slightly at acidic pH in mesothelioma cells (Table II).

Expression levels of selected genes in human cancers. Specimens from patients with lung, colon, stomach, liver and renal cancer in the Chiba Cancer Center Tissue Bank were available for the study. The homogenates of specimens from patients with lung cancer were not used due to a huge amount of skeletal material, so the measurement of gene expression was not assessed. Therefore, the expression of 8 selected genes was examined in the specimens from patients with colon, stomach, liver and renal cancer.

The specimens from the colon, stomach and renal cancer tissues showed increased *MnSOD*, *IL-32* and *TNFRSF9* transcripts compared to those from the non-tumorous regions of the same patients (Fig. 1). Increased expression of *AREG* was found in colon and renal cancer specimens (Fig. 1). Notably, an elevated expression of *ATP6V0D2* was found in stomach cancer specimens, whereas the expression was reduced in the specimens from patients with colon and renal cancer (Fig. 1). The expression of *ErbB3* was shown to be higher in colon, stomach and liver cancer specimens compared to the normal tissues, but a higher expression was observed in less than half of the renal cancer samples (Fig. 1). An increased expression of *LOC553158* was found in the specimens from the colon and stomach cancer nests, but the expression decreased in the liver and renal cancer specimens (Fig. 1). The expression of *DMGDH* was upregulated in the specimens from the colon cancer tissues, and the upregulated expression was observed in about half of the samples from the patients with stomach, liver and renal cancer (Fig. 1).

Discussion

For >30 years, it has been well known that cancer nests are acidified. However, thus far, few *in vitro* studies using acidic medium to develop cancer markers and medicines for cancer therapies have been performed. Our previous studies suggested that *in vitro* screening of compounds with anti-proliferation activity in an acidic medium was useful for developing anti-cancer drugs (11). A >2-fold increase in expression was found in ~700 genes in mesothelioma cells as the medium was acidified (16). Mesothelioma is one cancer that is hard to treat and remains asymptomatic even at a late stage.

In the present study, the expression of 8 genes with acidosis-induced expression in mesothelioma cells were examined in human specimens from various cancers and

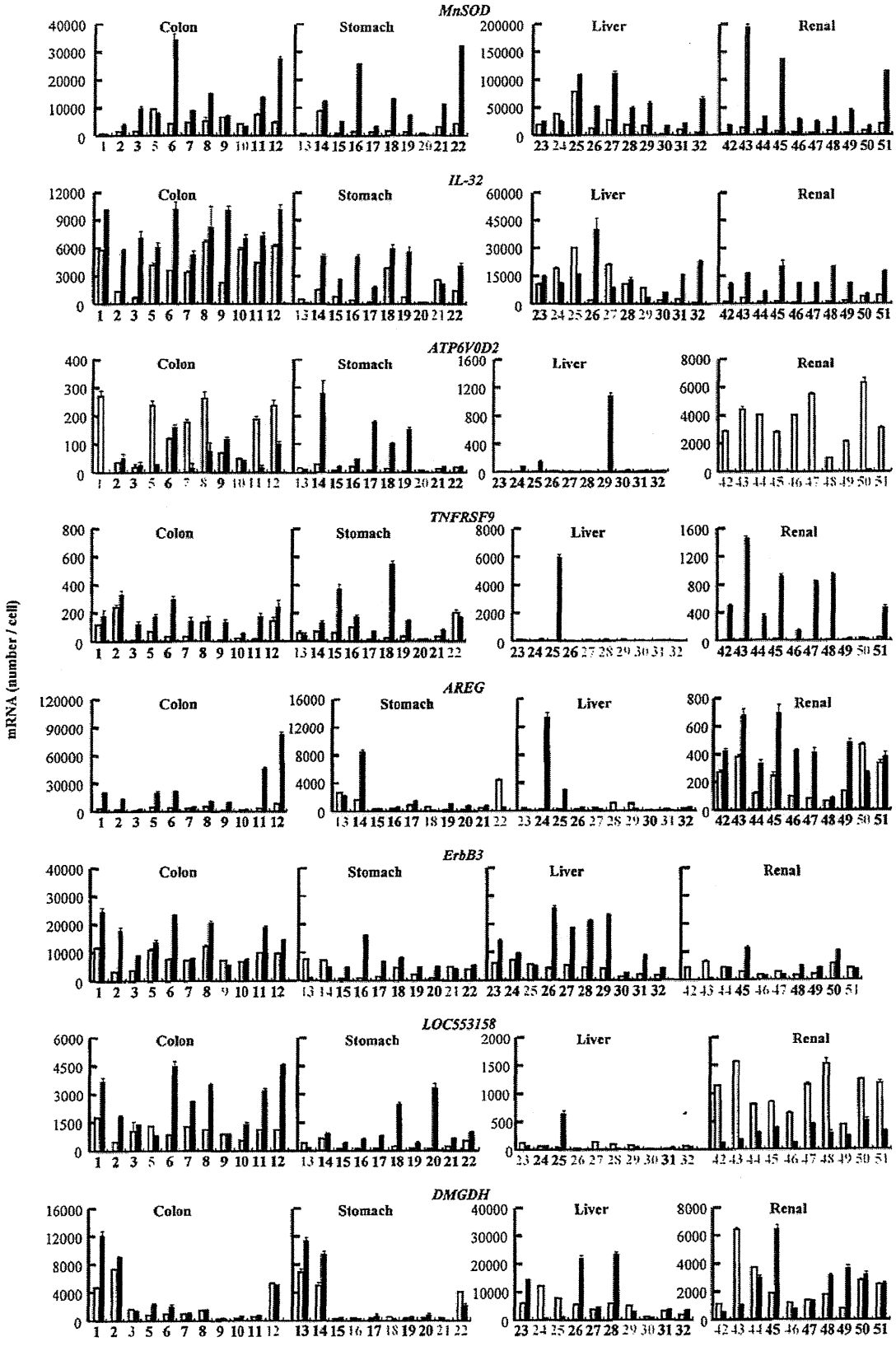

Figure 1. Gene expression in cancer tissues. RNA was extracted from human tumor (closed bars) and the corresponding non-tumorous tissues (open bars). The mRNA levels (*MnSOD*, *IL-32*, *ATP6V0D2* and *TNFRSF9*; *AREG*, *ErbB3*, *LOC553158* and *DMGDH*) were measured as described in the Materials and methods. The averages and standard deviation values were obtained from three experiments. The numbers in the horizontal axes correlate to the patient numbers. Grey numbers are the patients in which the gene expression decreased in the cancer tissues. *MnSOD*, manganese superoxide dismutase; *IL-32*, interleukin 32; *ATP6V0D2*, lysosomal H^+ transporting ATPase, V0 subunit d2; *TNFRSF9*, tumor necrosis factor receptor superfamily, member 9; *AREG*, amphiregulin, schwannoma-derived growth factor; *ErbB3*,v-erb-b2 erythroblastic leukemia viral oncogene homolog 3; *LOC553158*, PRR5-ARHGAP8; *DMGDH*, dimethylglycine dehydrogenase.

corresponding normal tissues. The expression varied in different tissues and showed a large variation among patients (Fig. 1). There may be a possibility that the genes that are specific to acidosis are expressed in a normal tissue area close to cancer nests as such an area may be acidified even if it contains no cancer cells. However, it is difficult to measure the pH of normal tissues prior to surgery as it can change during surgery due to the limited supply of blood. Furthermore, the pH may vary in different areas of cancer nests. In particular, the areas far from blood vessels are strongly acidified as suggested previously (2). Even though the data showed a wide variation, the present study produced several noteworthy results.

IL-32, *TNFRSF9*, *AREG*, *ErbB3*, *LOC553158* and *DMGDH* were expressed at a higher level than that of the normal areas in almost all the colon cancer patients. *MnSOD*, *IL-32*, *ATP6V0D2*, *TNFRSF9* and *LOC553158* were expressed at a higher level compared to the normal areas in almost all the patients with stomach cancer. Therefore, these genes may be candidate therapeutic or diagnostic marker targets for these cancers, and a combination use of these genes may be particularly useful for future treatment. In the liver cancer area, *MnSOD* and *ErbB3* were expressed at a higher level, but the expression of other genes was different in various patients. The reason for these differences in expression change remains unclear. Liver cancer nests may only be slightly acidified due to the highly organized blood vessel network in the liver.

IL-32 is a notable cytokine. This cytokine has been indicated to have a role in immune responses (21). The present data indicates that IL-32 is an interleukin that is specific to acidic conditions. As the mRNA level of *IL-32* was high in mesothelioma cells cultured at acidic pH ($2.6x10^5$ copies/cell, calculated from the data shown in Table II) and the numerous cancer nests measured in the present study (Fig. 1), this interleukin may be a predominant candidate for cancer diagnosis as indicated recently (22). TNFRSF9 has been suggested to play significant roles in immune responses (23). Our previous study demonstrated that the expression of *TNFRSF9* is induced in mesothelioma cells cultured in acidic media (16) and numerous cancer specimens (Fig. 1). Immune cells have to infiltrate into cancer nests or inflammatory loci to rehabilitate damaged tissues. Since cancer and inflammatory areas are often acidified, IL-32 and TNFRSF9 may function under acidic conditions in various cells besides the immune cells.

The ErbB/HER family, HER1 (epidermal growth factor receptor), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4), has been indicated to have a central role in a wide variety of growth factor-dependent cell responses (24). This family has been shown to mediate differentiation in neuroblastoma (25), and a high expression of *ErbB3* was found in neuroblastic tumors (26). High expression of *ErbB3* was also found in various cancers, and *ErbB3* has been identified as an attractive therapeutic target (27). Taken together with the present data, it can be argued that the gene product of *ErbB3* protects against cell death under acidic conditions. *AREG* was found to be expressed at high levels in colon and renal cancers, suggesting a role in carcinogenesis (28,29). To the best of our knowledge, this is the first study to report the expression of *LOC553158* itself in cancer cells, but the upregulation of *ARHGAP8* has been reported in cervical cancer (30).

DMGDH is a mitochondrial enzyme that has a role in choline catabolism [NCBI data base (31)]. No data concerning the role of DMGDH in carcinogenesis has been reported until the present study, and furthermore, no data to show the activation of the mitochondrial function in cancer cells has been reported. The present data indicate that choline catabolism may be activated in cancer areas or that DMGDH may mediate an unidentified metabolic process under acidic conditions besides choline catabolism.

The expression pattern of *ATP6V0D2* in renal tissues was unique. High expression of this gene was detected in normal areas, whereas almost no expression was observed in the cancer areas of all the patients. Protons are extruded to urine (32), and therefore, urine is often acidified. A high expression of *ATP6V0D2* has been previously reported in normal renal tissues (33). Therefore, it is quite possible that this gene is expressed in normal renal tissues to protect cells against external acidosis. The function to extrude protons may be diminished during carcinogenesis, resulting in the attenuation of this gene expression.

The genes with elevated expression levels in cancer specimens as compared to the surrounding normal tissues may be good candidates as novel targets and markers for cancer therapy. Particularly, a combination therapy may be more useful for the diagnosis of carcinogenesis and chemotherapeutics against cancer. The expression of 8 genes with high expression in cells cultured at an acidic pH were examined and it was found that the gene expression was elevated in human cancer tissues in the present study. Further studies of other acidosis-dependent gene expressions to promote the development of novel cancer markers and/or chemotherapeutic targets are warranted in future studies.

Acknowledgements

The authors would like to express their appreciation to Chiba Cancer Center Tissue Bank (Japan) for providing the human specimens. We thank Dr Xin Wang for her contribution to this section of the study.

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Suzuki et al. BMC Cancer 2014, 14:713
http://www.biomedcentral.com/1471-2407/14/713

BMC Cancer

RESEARCH ARTICLE Open Access

Mesenchymal stem cells are efficiently transduced with adenoviruses bearing type 35-derived fibers and the transduced cells with the IL-28A gene produces cytotoxicity to lung carcinoma cells co-cultured

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Abstract

Background: Transduction of human mesenchymal stem cells (MSCs) with type 5 adenoviruses (Ad5) is limited in the efficacy because of the poor expression level of the coxsackie adenovirus receptor (CAR) molecules. We examined a possible improvement of Ad-mediated gene transfer in MSCs by substituting the fiber region of type 5 Ad with that of type 35 Ad.

Methods: Expression levels of CAR and CD46 molecules, which are the major receptors for type 5 and type 35 Ad, respectively, were assayed with flow cytometry. We constructed vectors expressing the *green fluorescent protein* gene with Ad5 or modified Ad5 bearing the type 35 fiber region (AdF35), and examined the infectivity to MSCs with flow cytometry. We investigated anti-tumor effects of MSCs transduced with *interleukin (IL)-28A* gene on human lung carcinoma cells with a colorimetric assay. Expression of IL-28A receptors was tested with the polymerase chain reaction. A promoter activity of transcriptional regulatory regions in MSCs was determined with a luciferase assay and a tumor growth-promoting ability of MSCs was tested with co-injection of human tumor cells in nude mice.

Results: MSCs expressed CD46 but scarcely CAR molecules, and subsequently were transduced with AdF35 but not with Ad5. Growth of MSCs transduced with the *IL-28A* gene remained the same as that of untransduced cells since MSCs were negative for the IL-28A receptors. The *IL-28A*-transduced MSCs however suppressed growth of lung carcinoma cells co-cultured, whereas MSCs transduced with AdF35 expressing the *β-galactosidase* gene did not. A regulatory region of the *cyclooygenase-2* gene possessed transcriptional activities greater than other tumor promoters but less than the cytomegalovirus promoter, and MSCs themselves did not support tumor growth *in vivo*.

Conclusions: *AdF35 is a suitable vector to transduce MSCs that are resistant to Ad5-mediated gene transfer. MSCs infected with AdF35 that activate an exogenous gene by the cytomegalovirus promoter can be a vehicle to deliver the gene product to targeted cells.*

Keywords: Mesenchymal stem cells, Adenovirus, Type 35 adenovirus fiber, IL-28A

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Background

Bone marrow-derived mesenchymal stem cells (MSCs) have ability to differentiate into many kinds of tissues under a certain condition [1,2]. The pluripotency as progenitor cells indicates a potential clinical utility in multiple areas including tissue engineering. Furthermore, MSCs tend to migrate into inflammatory regions, damaged areas and tumors [3,4], which increases applications of MSCs as a tool to deliver an agent to target tissues and cells. Gene and cell therapy can be one of the directions to use MSCs as a cellular vehicle that distributes a therapeutic gene product into target cells and tumors. Administration of transduced MSCs in the vicinity of tumors can transport the gene product into the microenvironment as well.

Adenoviruses (Ad)-mediated transduction is one of the efficient methods to transfer an exogenous gene into human cells. The transduction efficacy with Ad vectors is however influenced by expression levels of the receptor molecules on target cells [5]. Attachment of type 5 Ad (Ad5), commonly used in a gene transfer system, to cells is mediated primarily by the binding of Ad fibers, which include the shaft and the knob regions, to the cellular receptor, the coxsackie adenovirus receptor (CAR) molecules, and secondly by the interaction between Ad penton bases and integrin molecules [6]. Expression levels on CAR molecules are dependent on respective cells and are often down-regulated in human tumors, which resulted in poor transduction efficacy in CAR-low cells [7]. On the other hand, subgroup B Ad such as type 35 use CD46 molecules as one of the major receptors and infect cells in a CAR-independent manner [8]. CD46 is expressed in a variety of human cells and the expression levels were not down-regulated in tumors. Recombinant Ad5 of which the fiber region is replaced with that of type 35 Ad (AdF35) can therefore infect cells in a similar manner as type 35 Ad through the type 35-derived fibers, which may widen a scope of target cells that are restricted by non-ubiquitous CAR distributions.

A new class of interferon (IFN), type III IFNs comprising of IFN-λ1, -λ2 and -λ3 which are also known as interleukin-29 (IL-29), IL-28A and IL-28B, respectively, has a similar biological functions as type I IFNs such as IFN-α and IFN-β [9,10]. The receptor complex of type III IFNs is composed of the IL-10 receptor beta (IL-10Rβ) and a novel IL-28 receptor alpha (IL-28Rα). In contrast to ubiquitous expression of IL-10Rβ, IL-28Rα expression is restricted to be tissue-specific, which subsequently confines the biological activities in IL-28Rα positive cells. The type III IFNs produce an anti-proliferative activity and induce apoptosis to a certain type of the receptor positive tumors including lung carcinoma [11] and esophageal carcinoma [12]. Moreover, several studies demonstrated that type III IFNs expressed in tumors achieved anti-tumor effects *in vivo* and some of the effects were mediated by non-immune mechanisms including anti-angiogenesis and by immunological responses such as activation of natural killer cells and dendritic cells [13-17].

In this study, we examined infectivity of Ad5 and AdF35 to human MSCs and investigated a possible use of MSCs as a vehicle to deliver gene products to tumors. We transduced MSCs with the *IL-28A* gene using a replication-incompetent AdF35 vector and tested whether the transduced MSCs produced cytotoxicty to tumor cells co-cultured. We also examined promoter activities in MSCs regarding transcriptional regulatory regions of the genes which are preferentially activated in human tumors.

Methods

Cells and mice

Human embryonic kidney HEK293 cells, human esophageal carcinoma YES-2 and TE-11 cells, human lung carcinoma OBA-LK1 cells, human immortalized fibroblasts OUMS-24 [18] and HFF cells [19], were cultured with RPMI1640 cells supplemented with 10% fetal bovine serum. MSCs derived from human bone marrow (PT-2501) (Cambrex, Rutherford, NJ, USA) were maintained with Mesenchymal Stem Cell Basal Medium (MSCBM; Cambrex). BALB/c *nu/nu* mice were purchased from Japan SLC (Hamamatsu, Japan).

Flow cytometry for receptor expression

Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD46 antibody (Ab) (BD Bioscience, San Jose, CA) or FITC-conjugated isotype-matched control Ab (BD Biosciences) as a control, or were reacted with anti-CAR (Upstate, Lake Placid, NY, USA), anti-CD51 (Chemicon, Temecula, CA, USA), anti-αvβ3 (Chemicon) or anti-αvβ5 Ab (Abcam, Cambridge, MA, USA) followed by FITC-conjugated goat anti-mouse IgG Ab (Kirkegaard & Perry, Gaithersburg, MD, USA). They were then analyzed for the fluorescence intensity with FACSCalibur (BD Bioscience) and CellQuest software (BD Bioscience).

Construction of Ad vector

The *green fluorescent protein* (GFP), the *β-galactosidase* (LacZ), the human *IL-28A* genes were cloned into pShuttle 2 (Takara Bio, Tokyo, Japan) and then ligated with Adeno-X vector (Takara Bio) of which the fiber region was replaced with that of type 35 Ad. The fiber modified Ad DNA was produced by inserting the Eco RI fragment containing the type 35 Ad fiber region (Avior therapeutics, Seattle, WA) (AY271307 at 30827–33609) into the corresponding site of Adeno-X vector DNA. The fiber modified Ad expressing the above genes, AdF35-GFP, AdF35-LacZ, and AdF35-IL-28A, and type 5 Ad bearing the GFP gene (Ad5-GFP) were produced by transfecting

the respective DNA into HEK293 cells and purified with an Adeno-X virus purification kit (BD Biosciences).

Infectivity of Ad

Cells were infected with Ad5-GFP or AdF35-GFP at multiplicity of infection (MOI) of 3 or 30 for 30 min and were washed to remove Ad. Infected cells were cultured for 2 days and then analyzed for percentages of GFP-positive cells with FACSCalibur and CellQuest software. Cells of which fluorescence was greater than the brightest 5% of uninfected cells were judged as positively stained.

Reverse transcription-polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and amplification of equal amounts of the cDNA was performed with the following primers and conditions: for the *IL-28Rα* gene, 5'-GGGAACCAAGGAGCTGCTAT G-3' (sense) and 5'-TGGCACTGAGGCAGTGGTGT T-3' (anti-sense), and 10 sec at 94°C for denature/20 sec at 58°C for annealing/28 cycles; for the *IL-10Rβ* gene, 5'-TATTGGACCCCCTGGAAT-3' (sense) and 5'-GTA AACGCACCACAGCAA-3' (anti-sense), and 10 sec at 94°C/20 sec at 50°C/28 cycles; for the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene, 5'-ACCAC AGTCCATGCCATCAC-3' (sense) and 5'-TCCACCAC CCTGTTGCTGTA-3' (anti-sense), and 15 sec at 94°C/ 15 sec at 60°C/25 cycles.

Cytotoxic test and enzyme-linked immunosorbent assay (ELISA)

OBA-LK1 cells were cultured in 96-well plates with MSCs uninfected or infected with AdF35-IL-28A or AdF35-LacZ (MOI = 100), at a ratio of 10: 1 or 10: 3. Cell viabilities after 4-days culture were assayed with a WST kit (Dojindo, Kumamoto, Japan) which detected the amounts of formazan produced from the WST-8 reagent with the absorbance at 450 nm (WST assay). OBA-LK1 cells were also labeled with PKH 26 dye according to the manufacturer's protocol (Sigma-Aldrich, St Louis, MO, USA) and cultured with MSCs, uninfected or infected with AdF35-IL-28A or AdF35-LacZ (MOI = 100), at a ratio of 10: 1 or 10: 3 for 4 days. They were then stained with Hoechst 33342 dye (Molecular Probes, Eugene, OR, USA) and numbers of PKH 26 positive and Hoechst 33342 positive cells were counted with confocal microscope (Olympus, Tokyo, Japan). The amounts of secreted IFN-28A were determined by an ELISA kit (R&D Systems, Minneapolis, MN).

Dual luciferase assay

Genomic fragments containing a transcriptional regulatory region of the *midkine* (0.6 kb, GenBank: D10604) [20], the *survivin* (0.5 kb, GenBank: U75285) [21], or the *cyclooxygenase-2* (0.3 kb, GenBank: U04636) gene [22] were cloned into pGL-2 basic vector (Promega, Madison, WI, USA) that contained the *firefly luciferase* gene. Plasmid DNA containing the respective genomic fragments, pGL-control vector (Promega) harboring the SV40 T antigen promoter-linked *firefly luciferase* gene, pGL-2 basic vector containing the cytomegalovirus (CMV) promoter or pGL-basic vector without any transcriptional regulatory regions (Promega), and a control vector, the *renilla luciferase* gene fused with the *herpes simplex virus-thymidine kinase* gene promoter (pRL-TK, Promega), at a molar ratio of 10: 1, was transfected into MSCs with a lipofectin reagent (Life Technologies, Gaithersburg). Cell lysate on day 2 was assayed for the luciferase activity with the dual luciferase reporter assay (Promega). The firefly luciferase activity was standardized by the amounts of luminescence produced by renilla luciferase and the relative activity was expressed as a percentage of the SV40 T antigen promoter-mediated activity.

Animal study

YES-2 cells (1×10^6) and MSCs or OUMS-24 cells at a ratio 5: 1 or 2: 1 (2×10^5 or 5×10^5) were inoculated subcutaneously into BALB/c *nu/nu* mice (6-week-old females). Tumor volume was calculated according to the formula ($1/2 \times \text{length} \times \text{width}^2$).

Statistical analysis

Statistical analysis was conducted with the one-way analysis of variance (ANOVA).

Results

Expression of Ad receptors on MSCs

We examined expression levels of CAR and CD46 molecules, the major Ad receptors of type 5 and type 35, respectively, on HEK293 cells and MSCs (Figure 1A). HEK293 cells, often used for Ad productions, expressed both receptors, whereas MSCs scarcely expressed CAR but were positive for CD46 expression. We also tested integrin molecules which were subsidiary receptors for type 5 Ad (Figure 1B). Both HEK293 cells and MSCs expressed CD51 that corresponded to integrin αv chain, αvβ3 and αvβ5 molecules. We calculated relative expression levels of these receptor molecules on MSCs in comparison with HEK293 cells and showed that MSCs expressed poorly CAR, moderately CD46 and well integrin molecules (Figure 2).

Infectivity of Ad5 and AdF35 to MSCs

We investigated efficacy of Ad5- and AdF35-mediated transduction with respective Ad bearing the *GFP* gene (Figure 3A). HEK293 cells became GFP positive after transduction with either Ad5-GFP or AdF35-GFP, but MSCs expressed GFP only when transduced with AdF35-GFP (Figure 3B). Percentages of GFP positive HEK293