RESEARCH PAPER

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Integrin alpha9 (ITGA9) expression and epigenetic silencing in human breast tumors

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Integrin alpha9 (ITGA9) is one of the less studied integrin subunits that facilitates accelerated cell migration and regulates diverse biological functions such as angiogenesis, lymphangiogenesis, cancer cell proliferation and migration. In this work, *integrin alpha9* expression and its epigenetic regulation in normal human breast tissue, primary breast tumors and breast cancer cell line MCF7 were studied. It was shown that *integrin alpha9* is expressed in normal human breast tissue. In breast cancer, *ITGA9* expression was downregulated or lost in 44% of tumors while another 45% of tumors showed normal or increased ITGA9 expression level (possible aberrations in the *ITGA9* mRNA structure were supposed in 11% of tumors). Methylation of *ITGA9* CpG-island located in the first intron of the gene was shown in 90% of the breast tumors with the decreased *ITGA9* expression while no methylation at 5'-untranslated region of *ITGA9* was observed. 5-aza-dC treatment restored *integrin alpha9* expression in ITGA9-negative MCF7 breast carcinoma cells, Trichostatin A treatment did not influenced it but a combined treatment of the cells with 5-aza-dC/Trichostatin A doubled the *ITGA9* activation. The obtained results suggest CpG methylation as a major mechanism of *integrin alpha9* inactivation in breast cancer with a possible involvement of other yet unidentified molecular pathways.

Introduction

Integrins are transmembrane glycoprotein receptors responsible for cell-cell and cell-matrix interactions.[1,2] At the cell surface, integrins not only keep the structural organization of continuous matrix-cytoskeleton network but participate in cell signaling and regulation of cell proliferation, adhesion and migration.[3] In carcinogenesis, integrins play an important role in cancer angiogenesis[4] and metastasis,[5] revealing them as potential targets for novel anti-tumor and anti-angiogenesis therapies based on chemical inhibitors or antagonists against different integrin subunits.[6-8]

Structurally, integrins are heterodimer molecules composed of two transmembrane glycoprotein subunits α and β (α,β). Up to date, there are 18 α and 8 β integrin subunits forming 24 different integrin molecules with their own function in normal and pathological cell physiology.[9]

One of the less studied integrin subunits is *integrin alpha9* (*ITGA9*, NM_002207) initially cloned from the lung and colon human cDNA libraries.[10] Integrin alpha9 subunit interacts only with beta1 subunit generating α9β1 heterodimer, which is expressed in many cell types such as epithelial cells, neutrophiles, hepatocytes, muscle and endothelial cells.[10-13] *ITGA9* expression is essential for the vital activity of the organism; mice homozygous for a null mutation in the alpha9 subunit gene die between 6 and 12 d of age.[14]

Integrin α9β1 is a receptor for thrombospondin-1 (THBS1),[15] ADAM12/ADAM15[16] and nerve growth factor (NGF),[17] interacts with vascular cell adhesion molecule 1 (VCAM1),[18] fibronectin,[19] tenascin C,[20] osteopontin,[21] VEGF-C, -D[22] and VEGF-A[23] isoforms. Functionally, integrin α9β1 is involved in angiogenesis and lymphangiogenesis, proliferation and migration of the different cells[24-26] playing an important role both in normal physiology and different pathological processes including carcinogenesis.

Unfortunately, there are not so abundant data on the *integrin alpha9* (*ITGA9*) expression in tumor tissues. It was shown that *ITGA9* is expressed in melanoma cells,[27] aberrantly upregulated in small-cell lung cancers, both cell lines and primary tumors[28] and medulloblastoma cells,[29] the expression level of integrin α9β1 on astrocytomas is correlated with increased grade of this brain tumor and is highest on glioblastoma, whereas normal astrocytes do not express this integrin,[30] ITGA9 is absent in normal adults colon tissues but it is expressed in 6 of 10 primary colon adenocarcinomas and 2 of 7 colon adenocarcinoma cell lines (Caco-2 and T84).[31] According to another study, *ITGA9* has significantly higher expression levels in colorectal tumors with high microsatellite instability (11 tumors of 42) vs. colorectal tumors with low or null microsatellite instability (31 tumors of 42).[32] Molecular

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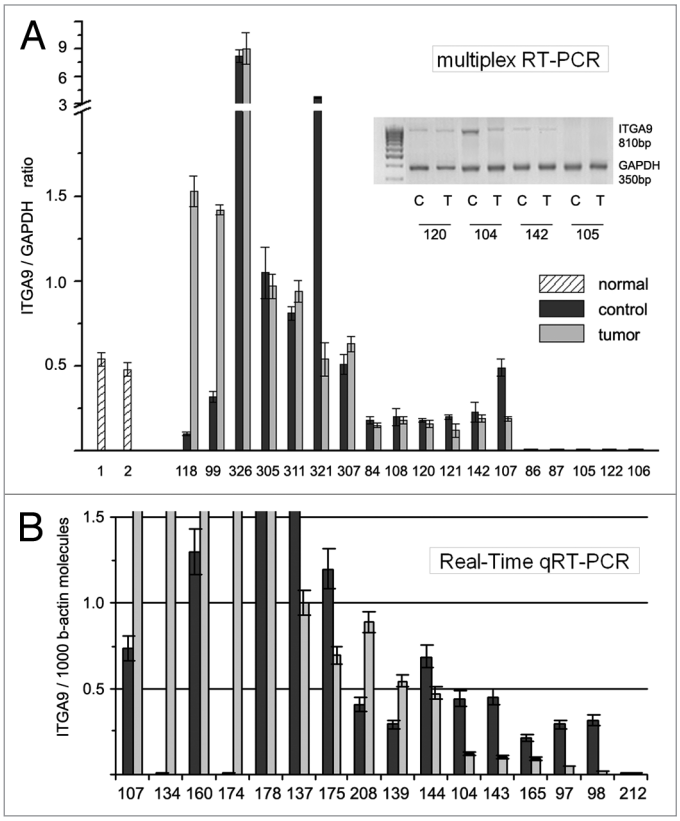

Figure 1. *ITGA9* expression in normal and tumor human breast tissue. (A) Multiplex RT-PCR. Intensity of the amplified *ITGA9* fragments normalized to that of GAPDH (TotalLab Programme). Representative gel from multiplex RT-PCR is shown (inset). (B) Quantitative Real-Time RT-PCR. ITGA9 expression normalized to 1000 b-actin molecules. The graph shows the mean expression levels from triplicate experiments (± SD) (OriginPro 8.1). 1 and 2, normal breast tissue; 84–32, breast tumors.

mechanisms of *ITGA9* activation in cancer cells remain unclear; however the effect may unfavorably contribute to the cancer prognosis supporting *ITGA9* as a potential target for anti-integrin therapy.

From the other side, extensive search of genetic/epigenetic aberrations of *ITGA9* by NotI-micorarray identified frequent (more than in 30%) aberrations (deletions, methylation) in kidney, lung, breast, ovary, cervical, prostate and colorectal cancer,[33,34] in 45–55% of head and neck squamous cell carcinomas[35] and 65% of uterine cervical carcinomas (41% deletion and 24% methylation).[36] A possibility of ITGA9 CpG-island methylation was verified in cervical[33] and colorectal[34] tumors by methyl-specific PCR and bisulfite sequencing.

In breast tumors, integrin α9β1 expression was shown immunohistochemically in 23 of 90 cases (26%)[37] but it is complicated to interpret the data in terms of increase/decrease of *ITGA9* expression in breast cancer because of absence of the data on its basic expression in normal human breast tissue.

In this study, we have investigated an *integrin alpha9* expression in normal human breast tissue, primary breast tumors and breast carcinoma cell line MCF7 and check a possible epigenetic regulation of *ITGA9* expression in breast cancer.

Results

ITGA9 expression in human breast tumors. Multiplex and TaqMan-based quantitative Real-Time RT-PCR analysis was used to determine *integrin alpha9* (*ITGA9*) expressions in human breast tumors with GAPDH and b-actin as the reference genes, respectively. Tumor and control samples were matched pairs for each patient with malignancy—from the central part of tumor and a more distant part of the breast, accordingly. Tissue samples obtained from the patients undergoing cosmetic surgery were designated as normal breast tissue. Two different primer pairs for different regions of the gene (amplified DNA fragments of 810 bp and 1100 bp) (Materials and Methods and **Fig. 3A**) were used to study *ITGA9* expression by multiplex RT-PCR. Two normal breast samples and 38 matched pairs (tumor and control) were analyzed (**Fig. 1**).

According to multiplex RT-PCR, the *ITGA9* was expressed in normal human breast tissue (**Fig. 1A**). However, in breast tumors, *ITGA9* expression was heterogeneously changed—there were both samples with significantly increased or decreased (up to complete disappearance) *ITGA9* expression level. These results were confirmed by TaqMan-based qRT-PCR for the same breast tumor and normal clinical samples and additional 20 matched pairs were studied (**Fig. 1B**). To analyze the obtained data, more than 2-fold expression change was taken as reliable in comparative analysis of *ITGA9* expression in tumor and control breast tissues. It was found that *ITGA9* expression was normal or increased in 45% of breast tumors (17 of 38 samples) and decreased or absent in another 44% of tumors (**Fig. 2**).

It is important to note that for most of the analyzed samples, similar *ITGA9* expression levels were shown using both primers pairs. However, 11% of samples (4 of 38 samples) showed a different *ITGA9* expression in dependence from the primer pairs used (**Fig. 3**). It indirectly supports an involvement of genetic changes (deletions, mutations) or pathological alternative splicing in ITGA9 function in breast cancer. Also, the fact underlines the importance of experimental design and primers/antibodies choice in *ITGA9* investigation because it could contribute to the obtained results.

Methylation status of ITGA9 CpG-island in breast tumors. On the next step, DNA methylation status of ITGA9 CpG-island (located at the first intron of the gene) was assessed by methyl-specific PCR (MSP) and bisulfite sequencing. In total, 12 breast tumors with decreased or absent ITGA9 expression and one tumor with normal expression level (sample 307) were studied (**Fig. 4**).

Hypermethylation of ITGA9 GpC-island located in the first intron of the gene was shown in 90% of the breast tumors with the decreased *ITGA9* expression (**Fig. 4A**) while no methylation at 5'-untranslated region of ITGA9 was observed in the same samples. Control sequencing of the DNA fragments amplified with methyl-specific primers and bisulfite sequencing confirmed high methylation of the CpG-island in these samples (although there were unmethylated clones as well possibly due to the presence of normal cells in the total tumor clinical sample) (**Fig. 4B**).

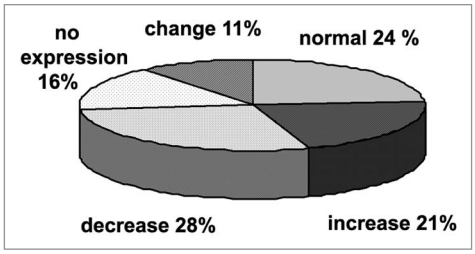

Figure 2. Diagram of subdivision of patients for different groups in dependency of *ITGA9* expression change in breast tumors.

The obtained results suggest hypermethylation of CpG-island as a main molecular mechanism of downregulation of ITGA9 expression in breast tumors. However, the presence of ITGA9 expression in some heavy-methylated breast tumors (samples 307 and 326) and its complete absence in tumor with non-methylated CpG region (sample 322) indicate a complexity of the process and an existence of another molecular mechanisms contributing to the regulation of *ITGA9* expression in breast cancer.

Restoration of ITGA9 expression in breast carcinoma MCF7 cells in vitro. To confirm hypermethylation as a main regulatory mechanism for ITGA9 inactivation in breast cancer, experiments on ITGA9 re-activation in breast cancer cells MCF7 in vitro were done.

At the first step, we showed that MCF7 cells practically do not express ITGA9 and CpG-island of the gene is almost completely methylated in the cells according bisulfite sequencing (data not shown). For the functional experiment in vitro, MCF7 cells were treated with DNA demethylating agent 5-deozyazacytidine (5-aza-dC) or hystone-deacetylase inhibitor Trichostatin A (TSA) or both. *ITGA9* expression level was then determined by multiplex RT-PCR with the primers for 810 bp amplified DNA fragment (**Fig. 5**).

It was shown that the 5-aza-dC treatment activated the ITGA9 expression in breast cancer cells MCF7 (near the 10-fold) but TSA treatment did not influence the expression. Combined 5-aza-dC/TSA treatment increased the activating effect up to 20-fold supporting a possible involvement of some other molecular pathways in the regulation of *ITGA9* expression in breast cancer.

Taken together, our results indicate that *integrin alpha9* expression is heterogeneously changed in human breast tumors and hypermethylation of ITGA9 CpG-island could be a major mechanism of *integrin alpha9* inactivation in breast cancer with a possible involvement of other unidentified yet molecular pathways.

Discussion

One of the important results of the study is detection of *ITGA9* expression in normal human breast tissue that was not shown earlier. The fact is of significance in its own right and logically corresponds to the literature data on *ITGA9* expression in different cell types—the integrin subunit is expressed in epithelial and smooth muscle cells,[10] on neutrophils[12,18] and human

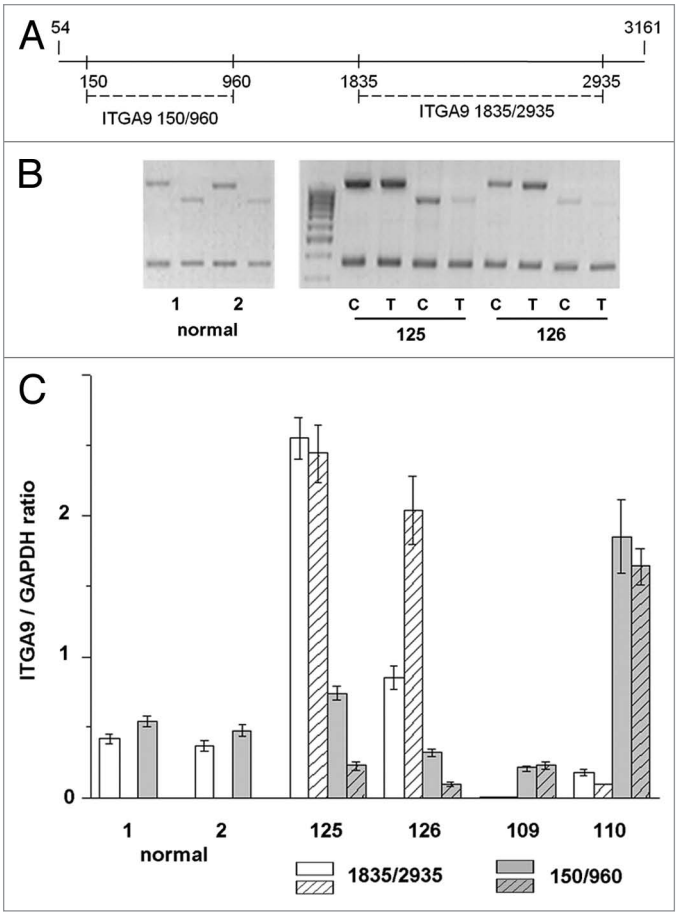

Figure 3. Multiplex RT-PCR analysis of *ITGA9* expression with different primer pairs. (A) Scheme of the primers used in the study. (B) Representative gel from multiplex RT-PCR. DNA fragments amplified with two different primers pairs are shown. *GAPDH* expression was used as an internal standard. (C) *ITGA9* expression levels normalized to that of *GAPDH* (TotalLab Programme). The graph shows the mean expression levels from triplicate experiments (± SD) (OriginPro 8.1). 1 and 2, normal breast tissue samples; 109, 110, 125 and 126, breast tumors; C and T, control and tumor breast tissue (match pair for each patient), respectively.

polymorphonuclear leukocytes,[13] which are constituent parts of breast tissue clinical sample. Thus, an expression of ITGA9 in normal breast tissue could be stated as a baseline to estimate its possible changes in different pathological conditions.

Interestingly, different levels of *ITGA9* expression in the control breast samples (normal counterparts of the matched pairs) were shown. There were both samples with the increased and decreased *ITGA9* expression, and not always it coincided with *ITGA9* expression in the tumor counterpart. Possibly, the control breast tissues are already affected by the disease or inversely, some pre-tumor changes occur in the breast tissue. The results suppose that a possibility to use a normal-looking counterpart as a normal tissue (in comparative study) should be preliminary tested for each experimental system.

According our RT-PCR data, *ITGA9* expression in breast tumors is changed bi-directionally—21% of tumors showed significantly increased *ITGA9* level and 44% of the studied tumors showed decreased *ITGA9* expression (up to complete absence).

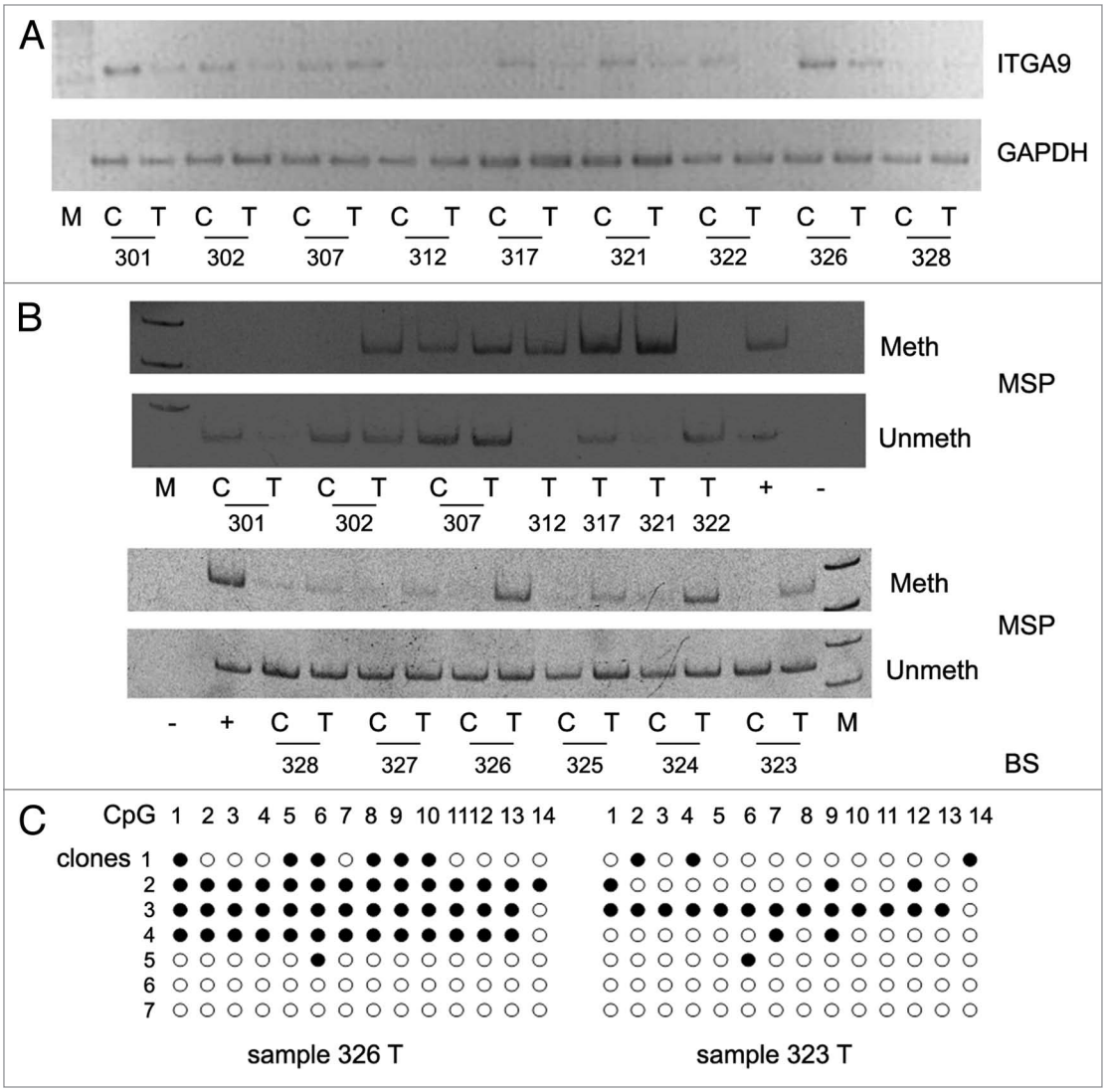

Figure 4. Methylation of ITGA9 CpG-island in primary human breast tumors. (A) ITGA9 expression in breast tumors; a representative gel from multiplex RT-PCR. (B) Methyl-specific PCR on *ITGA9* CpG-island. (C) Bisulfite sequencing. 301–328, breast tumors; C and T, control and tumor breast tissue (match pair for each patient); +, positive PCR control; –, negative PCR control; M, DNA marker; Meth and Unmeth, primers for methylated or un-methylated DNA sequence, respectively.

The obtained results supplement the data on *ITGA9* expression in 26% of breast tumors showed by immunohistochemistry.[37]

Here, two different aspects of the changes are to be discussed. From the one side, an activation of *ITGA9* expression was shown for different human tumors and cancer cells—small-cell lung cancer,[28] medulloblastoma cells,[29] astrocytomas and glioblastoma.[30] From the other side, frequent genetic/epigenetic aberrations (deletions, methylation) of *ITGA9* were identified in different epithelial cancers using NotI-microarray.[33-36] Taken together, the presented data suppose an existence of different molecular pathways participating in the regulation of *ITGA9* expression in cancer and resulting in the heterogeneity of primary tumors based on *ITGA9* expression level. In fact, it was shown for different cell lines in vitro that colon adenocarcinoma Caco-2 and T84 cells express the integrin alpha9 subunit while the five other colon carcinoma cell lines tested were negative for its expression,[31] two glioblastoma cell lines, LN229 and LN18 are alpha9beta1 integrin positive and negative, respectively.[30]

Our results are in concordance with these data and show that along with activation of *ITGA9* expression in some breast tumors there are groups of patients with absent (16% of tumors) or significantly decreased (28% of tumors) *ITGA9* expression. If the first group seems to consist of the tumors with genetically eliminated ITGA9 (deletions, mutations), the second group of tumors should have a functional *ITGA9* gene and some epigenetic mechanisms are possibly involved in the attenuation of *ITGA9* expression in the cells. Earlier, ITGA9 CpG-island methylation was indeed shown in more than 30% of cervical[33] and colorectal[34] tumors. According to our data, hypermethylation of *ITGA9* CpG-island could be an important mechanism for *ITGA9* epigenetic inactivation in breast cancer responsible for its decreased expression in about 25% of primary breast tumors.

However the expression of *ITGA9* in the samples with high methylation of ITGA9 CpG-island (samples 307 and 326) suggests an involvement of some antagonistic molecular regulators of *ITGA9* expression as well. The hypothesis is supported by the further observation that the combined treatment of MCF7 cells with 5-aza-dC/TSA increased ITGA9 expression level even more (2-fold) compare with the aza-treatment alone. Possibly, an activation of some positive regulators of *ITGA9* by Trichostatin A (TSA) treatment contributes to the 5-aza-dC-stimulated *ITGA9* expression in MCF7 cells. Thus, the presented data suggest that some other molecular pathways could be involved in *ITGA9* regulation in cancer along with the hypermethylation of its CpG-island.

A similar complex regulation of gene expression was shown for another member of the same integrin subfamily integrin alpha4beta1—aberrant DNA methylation of its promoter region results in integrin alpha4 silencing in 55% of cholangiocarcinomas,[38] 84.7% of 46 primary gastric tumors and 8 of 9 gastric cancer cell lines[39] while TSA treatment upregulated integrin alpha4 expression in hepatocellular carcinoma cell line Hep3B.[40]

The obtained data show complexity of ITGA9 regulation in breast cancer cells that result in existence of ITGA9-expressing or ITGA9-non-expressing breast tumors. Because of the epigenetic inactivation of ITGA9 in the last group, one can assume that the patients will not benefit from anti-integrin therapy and epigenetics drugs (like anti-methylation agents or HDAC inhibitors) while the first group could be taken into consideration for those treatments. In these terms, ITGA9 expression level could be an important diagnostic marker for novel epigenetic drugs or anti-integrin-based antimetastatic therapies.

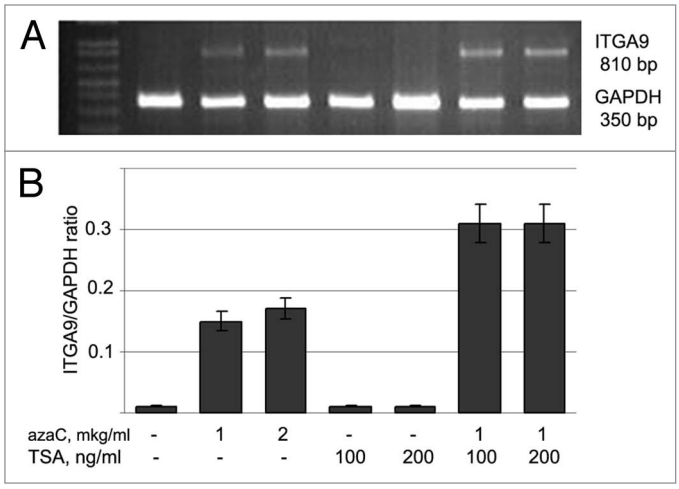

Figure 5. Activation of ITGA9 expression in MCF7 breast carcinoma cells by 5-deoxyazacytidine treatment. (A) Representative gel showing *ITGA9* amplified by multiplex RT-PCR. (B) Intensity of the amplified *ITGA9* DNA fragments normalized to that of *GAPDH* (TotalLab Programme). Bars represent the mean ± SD from triplicate experiments (OriginPro 8.1). The lane order on the gel corresponds to the bars on the histogram. 5-aza-dC, 5-azadeoxycytidine; TSA, Trichostatin A.

Materials and Methods

Reagents. TRIZOL reagent was from Invitrogen, M-MLV Reverse Transcriptase and RQ1 RNase free DNase were from Promega and 5-aza-dC and TSA were from Sigma.

Patients and tissue samples. A total of 38 patients with breast cancer and two individuals without malignancy (undergoing cosmetic surgery) were studied. All samples were obtained from primary breast tumors during the radical surgery at Central Municipal Hospital N1, Novosibirsk, Russia, "snap-frozen" in liquid nitrogen and stored at -70°C. Regions were manually dissected from the frozen blocks to provide a consistent tumor cell content of more than 70% in tissues used for analysis. The prevalent histological type of tumors was duct infiltrating cancer of different degree of malignancy. Most patients were at the second stage of malignancy progression according the formula $T_xN_xM_x$. All patients gave written informed consent. The study protocol has been approved by the Local Ethics Committee in accordance with the Helsinki Declaration of 1975.

RT-PCR analyses of ITGA9 expression. Total RNA was extracted from the cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. DNase treatment was done using RQ1 RNase free DNase I (Promega) at 37°C for 30 min with subsequent RNA precipitation with isopropanol. cDNA was synthesized from 1–2 μg of total RNA using oligo-dT primers and M-MTLV reverse transcriptase (Promega) according to the manufacturer's protocol and 1/10th of the product was subjected to PCR analysis.

The following conditions were used for multiplex RT-PCR: 94°C for 4 min, 94°C for 30 sec, 61°C (for ITGA9 1835/2935) or 64°C (for ITGA9 150/960) for 60 sec and 72°C for 1 min, with a final elongation step at 72°C for 10 min using a Tercik PCR machine (DNA-Technology). The total reaction volume was 10 μl. *ITGA9* 1100 bp and 810 bp DNA fragments were amplified for 39 and 37 cycles, respectively; *GAPDH* (housekeeping gene) was amplified for 27 cycles. The amplified products were separated on 1.0% agarose gels. The gels were scanned using the "DNA Analyzer" system and *ITGA9* expression levels were estimated from the intensity of the amplified *ITGA9* DNA fragment normalized against the intensity of *GAPDH* (TotalLab program, Nonlinear Dynamics). The PCR primers used for human *ITGA9* and *GAPDH* were as follows: ITGA9–1835/2935-F, 5'-CCT CTG ACA CCA GTT CTC CGC-3'; ITGA9–1835/2935-R, 5'-GCC TCG AAG ACC ACC GTC A -3'; ITGA9–150/960-F, 5'-GAC CCG CAG CGC CCC G-3'; ITGA9–150/960-R, 5'-GCG CAC AAG GAG GAG CCG-3'; GAPDH-F, 5'-GGG CGC CTG GTC ACA A-3'; GAPDH-R, 5'-AAC ATG GGG GCA TCA GCA GA-3'.

Quantitative real-time RT-PCR (qRT-PCR) was performed using the ABI PRISM 7000 Sequence Detector (AppliedBiosystems) and the *ITGA9* TaqMan Assay (Medigen) under the following conditions: 50°C for 2 min, 94°C for 10 min, followed by 40 cycles at 94°C for 15 sec and 60°C for 1 min. The total reaction volume was 25 μl. b-actin was used as the housekeeping gene. The PCR primers and TaqMan probes used were: ITGA9-F, 5'-GTT GGT GGG AAT CCT CAT CTT C-3'; ITGA9-R, 5'-TTT GTA CCT TCG GCG AAA GAA-3', ITGA9-probe, 5'-FAM- TGG CCG TGC TGC TCT

GGA AGA TG-TAMRA-3'; b-actin-F, 5'-GGC ACC CAG CAC AAT GAA G-3'; b-actin-R, 5'-GCC GAT CCA CAC GGA GTA CT-3'; b-actin-probe, 5'-FAM-TCA AGA TCA TTG CTC CTC CTG AGC GC-TAMRA-3'.

Genomic DNA isolation and bisulfite conversion. Genomic DNA was isolated from the tissue samples using E.Z.N.A. DNA isolation kit and bisulfite conversation of the genomic DNA was performed by E.Z.N.A. DNA methylation kit (Zymo Research) according to the manufacturer's instructions.

Methyl-specific PCR. Methyl-specific PCR for ITGA9 fragment amplification was performed with primers specific to the methylated (Met) and unmethylated (Unmeth) DNA sequences of *ITGA9* CpG island. Blood gDNA treated with SssI-methyltransferase (NewEngland Biolabs) was used as a positive control for Met-primers. The following conditions were used for PCR: 95°C for 4 min, 95°C for 30 sec, 60–65°C for 30 sec and 72°C for 30 sec, with a final elongation step at 72°C for 7 min, 45 cycles. Reaction mixture contained 1x DreamTaq buffer, 0.2 mM dNTPs, 0.4 μM primers, 3.5% DMSO, 50–100 ng of bisulfite converted DNA and 1.25 U of DreamTaq DNA-polymerase (Fermentas). The total reaction volume was 30 μl; 10 μl of the amplified products were separated on 10% PAAG and visualized by ethidium bromide staining.

The PCR primers were as follows: ITGA9-Met-F, 5'-TGG AGT ATT TTT ACG ATA ATA CGC-3'; ITGA9-Met-R, 5'-AAA AAC CGA AAA AAC GAC GA-3', (116 bp); ITGA9-Unmeth-F, 5'-TGG AGT ATT TTT ATG ATA ATA TGT GT-3'; ITGA9-Unmeth-R, 5'-AAA AAA AAC CAA AAA AAC AAC AAC-3', (119 bp).

Bisulfite sequencing. Amplification of the *ITGA9* DNA fragment for bisulfite sequencing was done using bisulfite-treated gDNA and primers specific for ITGA9 CpG-island sequence (ITGA9-F, 5'-CCC TGG GGT CCC AGC CCA GAG-3'; ITGA9-R, 5'-GAG AGG CTA TAC TCC TTC CTC AG-3'). The following conditions were used for PCR: 94°C for 2 min, 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min, with a final elongation step at 72°C for 10 min, 35 cycles. The total reaction volume was 30 μl. The PCR products were purified using a DNA Clean and Concentrator Kit (Zymo Research Corporation) and cloned in TOPO-vector using a TOPO TA Cloning Kit for Sequencing (Invitrogen BV) according to the manufacturer's instructions. Plasmid DNA was isolated using a Zyppy Plasmid Miniprep Kit (Zymo Research Corporation) according to the manufacturer's protocol. Sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction kit v1.1 and ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. Eight to 10 clones were analyzed for each sample.

Cell lines, cell culture and 5-aza-dC/TSA treatment. The MCF7 human breast cancer cell line was obtained from MTC (Karolinska Institute). Cells were maintained in Iscove's Modified Dulbecco's medium (IMDM) supplemented with \2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS at 37°C in a humidified 5% CO2 incubator. Deoxyazacytidine (5-aza-dC, 1 or 2 μg/ml) or Trichostatin A (TSA, 100 or 200 ng/ml) treatment was done by the incubation with the cells for 72 h or 24 h, respectively. For combined treatment, the cells were incubated with 5-aza-dC (1 μg/ml) for 48 h after that TSA (100 or 200 ng/ml) was added for additional 24 h. Cells were harvested for analysis using trypsin/EDTA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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