

The A₃ Adenosine Receptor Is Highly Expressed in Tumor *versus* Normal Cells: Potential Target for Tumor Growth Inhibition

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ABSTRACT

Purpose: A₃ adenosine receptor (A₃AR) activation was shown to inhibit the growth of various tumor cells via the down-regulation of nuclear factor κB and cyclin D1. To additionally elucidate whether A₃AR is a specific target, a survey of its expression in tumor *versus* adjacent normal cells was conducted.

Experimental Design: A₃AR mRNA expression in various tumor tissues was tested in paraffin-embedded slides using reverse transcription-PCR analysis. A comparison with A₃AR expression in the relevant adjacent normal tissue or regional lymph node metastasis was performed. In addition, A₃AR protein expression was studied in fresh tumors and was correlated with that of the adjacent normal tissue.

Results: Reverse transcription-PCR analysis of colon and breast carcinoma tissues showed higher A₃AR expression in the tumor *versus* adjacent non-neoplastic tissue or normal tissue. Additional analysis revealed that the lymph node metastasis expressed even more A₃AR mRNA than the primary tumor tissue. Protein analysis of A₃AR expression in fresh tumors derived from colon (*n* = 40) or breast (*n* = 17) revealed that 61% and 78% had higher A₃AR expression in the tumor *versus* normal adjacent tissue, respectively. The high A₃AR expression level in the tumor tissues was associated with elevated nuclear factor κB and cyclin D1 levels. High A₃AR mRNA expression was also demonstrated in other solid tumor types.

Conclusions: Primary and metastatic tumor tissues highly express A₃AR indicating that high receptor expression is a characteristic of solid tumors. These findings and

our previous data suggest A₃AR as a potential target for tumor growth inhibition.

INTRODUCTION

The A₃ adenosine receptor (A₃AR) belongs to the family of the G_i protein-associated cell surface receptors. Its activation induces inhibition of adenylyl cyclase and cyclic AMP formation, leading to the initiation of various signaling pathways, which are cell and stimulant specific (1). Our earlier studies showed that activation of A₃AR by the natural ligand adenosine or by synthetic agonists induces an inhibitory effect on tumor cell growth. The growth of melanoma, prostate, and colon carcinoma was markedly inhibited upon treatment with synthetic A₃AR agonists. The experimental murine models used included syngeneic models in which primary or metastatic melanoma (B16-F10) or colon carcinoma (CT-26) were inoculated, as well as xenograft models of colon and prostate carcinoma (HCT-116 and PC-3, respectively). IB-MECA and CI-IB-MECA prevented tumor growth in these models upon daily oral treatment. Mechanistic studies demonstrated that A₃AR activation induces down-regulation of key cell growth-regulatory proteins including cyclin D1 and nuclear factor κB (NFκB; Refs. 2–6). Interestingly, in normal murine bone marrow cells, A₃AR activation induced an opposite effect, *i.e.*, proliferation of myeloid cells (7, 8). These results led us to presume that the differential effect of A₃AR agonists on tumor and normal cells may be attributed to different expression level of the receptor in each cell type.

A₃AR expression was detected recently in tumor cell lines including astrocytoma, HL-60 leukemia, B16-F10 and A378 melanoma, human Jurkat T-cell lymphoma, and murine pineal tumor cells, whereas low expression was described in most body normal tissues (3–5, 9–13).

In the present study a comparison between A₃AR expression in tumor *versus* normal and metastatic human tissues was studied. In addition, we compared the A₃AR expression level with that of cyclin D1 and NFκB in colon and breast carcinoma *versus* the relevant normal tissues. Results show high receptor expression in the tumor and metastatic tissue *versus* low in the normal.

MATERIALS AND METHODS

Reagents. Rabbit polyclonal antibodies against human A₃AR was purchased from α Diagnostic (San Antonio, TX), human cyclin D1 from Upstate (Lake Placid, NY), and human NFκB from Chemicon (Temecula, CA). Goat polyclonal antibodies against β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Patient Samples. Paraffin-embedded slides for reverse transcription-PCR (RT-PCR) analysis were obtained from patients with the same class and stage of tumors, and the histopathological diagnosis is detailed in Table 1. For Western blot

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Table 1 Histopathological diagnosis of paraffin embedded slides from which mRNA was extracted

Tumor type	Histopathological diagnosis
Colon carcinoma	Differentiated adenocarcinoma of the colon, Dukes T2/T3 (colon/rectum)
Breast carcinoma	Infiltrating duct carcinoma grade III
Small cell lung carcinoma	Poorly differentiated adenocarcinoma of lung
Pancreatic carcinoma	Moderately differentiated ductal adenocarcinoma
Melanoma	Metastatic melanoma neoplasm in brain

and RT-PCR analysis, fresh tissue of colon and breast carcinoma was minced and transferred to liquid nitrogen. Colon carcinoma samples were obtained from patients with moderate differentiated adenocarcinoma of the colon, Dukes T2 or T3 of upper colon or rectum. Breast cancer samples were obtained from patients with infiltrating duct carcinoma grade III.

RT-PCR Analysis of Formalin-Fixed Paraffin-Embedded Tissue Slides. Tissue sections (5- μ m thick) were mounted on slides that were stained by H&E and were observed by a pathologist. The neoplastic area and the normal area were detected and marked each one separately. In each marked area cells were counted. Nonstained sequential slides were marked for neoplastic and normal tissue based on the stained slides. Tissue sections on slides were deparaffinized in xylene and rehydrated by washing in serial dilutions of ethanol. Slides were used immediately or stored at -80°C until used. After rehydration, 20 μ l of solution A [1.25 \times PCR buffer (200 mM Tris-HCl and 500 mM KCl), 6.25 mM MgCl_2 , 5 units RNasin (Promega, Madison, WI), 2 mM DTT, and 1 unit RQ1 RNase-free DNase (Promega)] was applied directly to the marked area. The marked area was completely scraped off the slide using a pipette tip, and the neoplastic tissue or the normal tissue was collected to different microcentrifuge tubes. The samples were treated with proteinase K at a final concentration of 0.1 mg/ml. The samples were incubated at 37°C for 1 h to allow for DNA digestion. Cells lysate were heated to 95°C for 15 min to inactivate DNase and proteinase K. After centrifugation at 14,000 rpm for 5 min, 17 μ l of the supernatant was transferred to separate tube, and 4 μ l of reverse-transcription mixture [5 mM deoxynucleoside triphosphate, 2.5 μ M random hexamer, 5 units RNasin, 100 units SuperScript One Step RT-PCR with Platinum Taq (Invitrogen), and the primers for $A_3\text{AR}$ 5'-ACGGTGAGGTACCACAGCT-TGTG and 3'-ATACCGGGATGGCAGACC] were added.

The reverse-transcription reaction was performed at 45°C for 45 min followed by heating to 99°C for 5 min. Next, 50 cycles of 94°C for 30 s, 59°C for 45 s, and 73°C for 45 s were performed. Products were electrophoresed on 2% agarose gels, stained with ethidium bromide (Et-Br), and visualized with UV illumination. The specificity of the RT-PCR reaction was confirmed by size determination on agarose gels in comparison with a positive control, from RNA extracted using standard techniques, and by sequencing the RT-PCR product and comparing the sequences to that of the known sequences (ADORA3-L77729, L77730). The absorbance of the bands (Et-Br) was quantified using an image analysis system. To quantitate $A_3\text{AR}$ mRNA expression, the absorbance value was normalized

against the cell number in each marked area (tumor or adjacent tissue).

RT-PCR Analysis of Fresh Tumor Tissue. The reverse-transcription reaction was performed at 45°C for 45 min from 1 μ g total RNA extracted using tri-reagent (Sigma). For $A_3\text{AR}$ amplification we used the same primers described above; the reverse transcriptase was followed by heating to 99°C for 5 min, 50 cycles of 94°C for 30 s, 59°C for 45 s, and 73°C for 45 s. For amplification of human β -actin the primers 5'-TGGGAATGGGTCAGAAGGACT and 3'-TTTCACGGTTGGCCTTAGGGT were used. The PCR condition included heating to 94°C for 2 min, 30 cycles of 94°C for 30 s, 56°C for 1 min and 30 s, and 73°C for 45 s.

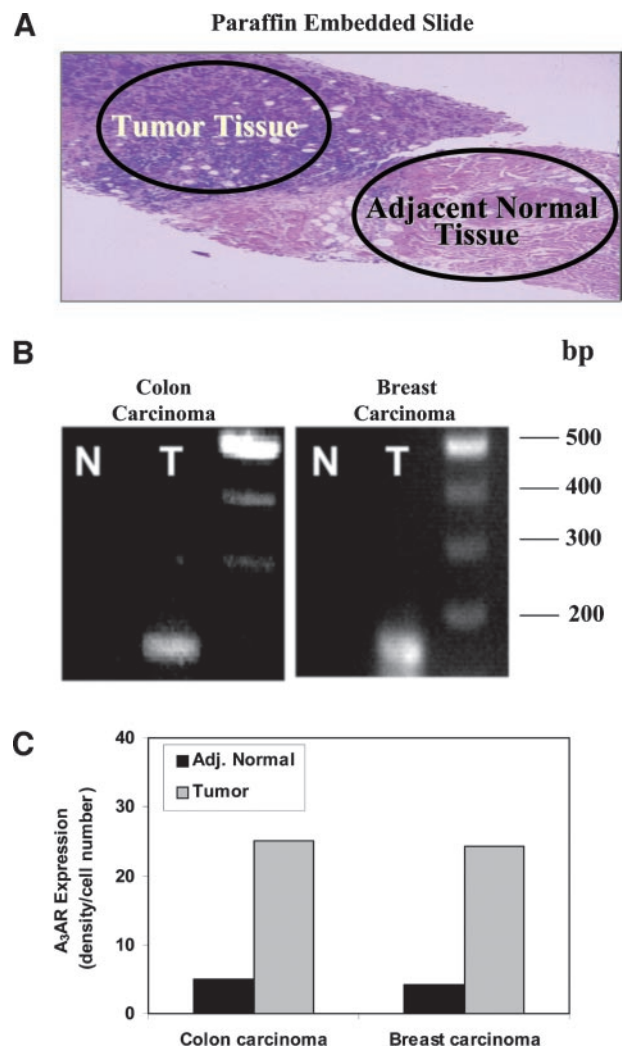


Fig. 1 A_3 adenosine receptor ($A_3\text{AR}$) mRNA expression in colon and breast carcinoma versus the adjacent normal tissue. A, formalin-fixed, paraffin-embedded slides were marked for the neoplastic and normal adjacent tissue. Analysis was carried out on tissue mRNA extracts derived from the unstained sequential slides. B, $A_3\text{AR}$ mRNA (reverse transcription-PCR) expression in colon and breast neoplastic versus adjacent normal tissue. C, quantitation of $A_3\text{AR}$ mRNA expression was based on the density (ethidium bromide) of $A_3\text{AR}$ expression in each specimen divided by cell count in the tumor or normal marked area.

Table 2 Summary of A₃AR^a mRNA expression level in pathological slides from colon and breast tumor and the relevant normal tissues

Tissue	Type	A ₃ AR + (n)	Score ^b				Mean score
			1	2	3	4	
Colon carcinoma	Normal	3 (10)	1		2		0.7
	Primary tumor	7 (7)	2	1	2	2	1.7
Breast carcinoma	Normal	2 (10)				2	0.8
	Primary tumor	10 (10)		5	2	3	1.8

^a A₃AR, A₃ adenosine receptor.

^b Formalin-fixed, paraffin-embedded slides were marked for the neoplastic and normal adjacent tissue. Analysis was carried out on tissue mRNA extracts derived from the unstained sequential slides. Score (1, 2, 3, or 4) for A₃AR mRNA was determined based on the density value (ethidium bromide) of mRNA in each specimen divided by the cell number. The mean score is the sum of the frequency of each specimen multiply by its respective score, divided by the total number of specimens.

Western Blot Analysis. To detect A₃AR expression level and additional cell growth-regulatory proteins Western blot analysis was performed. Tissue samples were homogenized by Ultraturax (Polytron, Kinematica), and protein was in ice-cold lysis buffer [TNN buffer, 50 mM Tris buffer (pH 7.5), 150 mM NaCl, and NP40 0.5% for 20 min]. Cell debris was removed by centrifugation for 10 min, at 14,000 rpm. The supernatants were used for Western Blot analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50 μg) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked with 1% BSA and incubated with the desired primary antibody (dilution 1:1000) for 24 h at 4°C. Blots were then washed and incubated with a secondary antibody for 1 h at room temperature. Bands were recorded using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium color development kit (Promega). The absorbance of the bands was quantified using an image analysis system and corrected by the absorbance of the corresponding actin bands. Data presented in the different figures are representative of at least three different experiments.

Statistical Analysis. Results were analyzed by Wilcoxon signed rank test, with statistical significance at $P < 0.05$. Score (1, 2, 3 or 4) for A₃AR mRNA expression level was determined based on the density values (Et-Br) of A₃AR expression in each specimen divided by cell number. Mean score was calculated according the following formula:

$$\frac{\sum n_i * \text{Score}_i}{n}$$

where n_i is the number of specimens with the same score.

RESULTS

Expression of A₃AR mRNA in Colon and Breast Carcinoma versus Adjacent Normal Tissue. In this set of experiments mRNA was extracted from formalin-fixed, paraffin-embedded tissue sections (5-μm thick). To differentiate between the neoplastic and normal regions, slides were stained with H&E and observed by a pathologist. Later on, nonstained sequential slides were marked for neoplastic and normal area based on the stained slides. Tissue was scarped from the marked area and collected separately to two different tubes for the RT-PCR

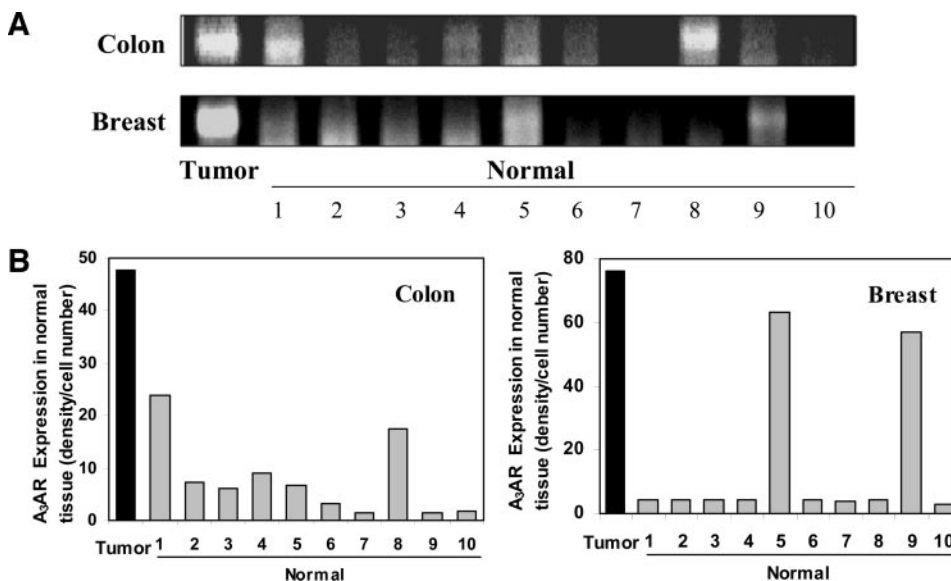


Fig. 2 A₃ adenosine receptor (A₃AR) mRNA expression (reverse transcription-PCR) in normal (nontumorigenic) colon and breast tissues (A). Formalin-fixed, paraffin-embedded slides were marked for the neoplastic and normal adjacent tissue. Analysis was carried out on tissue mRNA extracts derived from the unstained sequential slides. B, quantitation of A₃AR mRNA expression was based on the density (ethidium bromide) of A₃AR expression in each specimen divided by cell count in the tumor or the normal marked area in the slides.

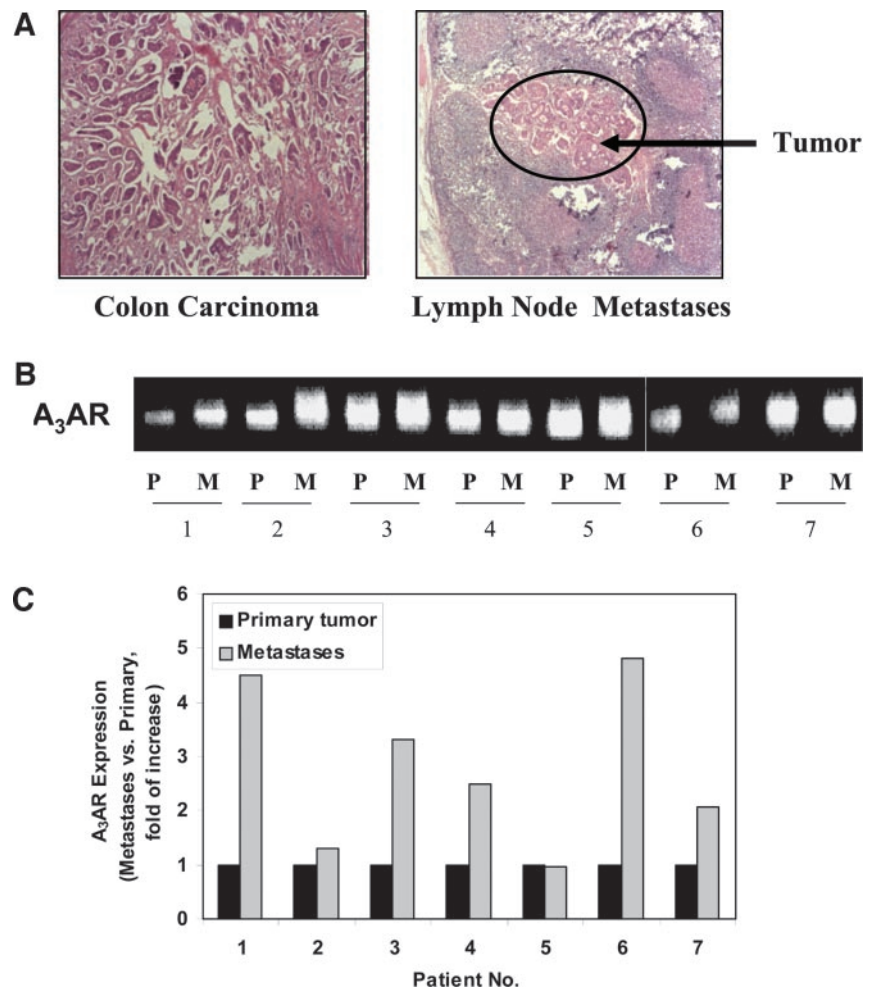


Fig. 3 A₃ adenosine receptor (A₃AR) mRNA expression in primary and metastases of colon carcinoma. **A**, slides mounted with primary or metastatic tissue were marked for the neoplastic area. **B**, mRNA was extracted from tissues of sequential slides and was used for reverse transcription-PCR analysis. **C**, quantitation of A₃AR mRNA expression based on the density (ethidium bromide) of A₃AR expression divided by the size of the area from which mRNA was extracted. The values derived from the metastatic lesion were normalized against the value derived from the primary lesion. *P*, primary tumor; *M*, metastasis.

reaction (Fig. 1A). In both colon and breast tissues, higher A₃AR mRNA expression was noted in the tumor in comparison with the adjacent normal tissue (Fig. 1, *B* and *C*; Table 2). A representative blot is depicted in Fig. 1B.

We additionally tested mRNA expression level in normal colon (diverticulitis) and breast tissues and compared it with a relevant representative sample of tumor tissue (Fig. 2A). Quantitation of A₃AR mRNA expression was based on the density (Et-Br) of A₃AR expression in each specimen. The density values are expressed as percentage of the density of A₃AR in the tumor tissue. A₃AR mRNA expression was lower in all of the tested normal tissues (Fig. 2B).

Comparison between A₃AR Expression in Primary versus Metastases in Colon and Breast Tissues. To elucidate the expression of A₃AR mRNA in primary versus metastases, we compared the level of A₃AR mRNA expression in primary and regional lymph node metastases of colon and breast in formalin-fixed, paraffin-embedded tissue (Fig. 3A and Fig. 4A). To quantitate A₃AR mRNA expression, the density (Et-Br) of the bands related to A₃AR were measured, and values were divided by area size from which mRNA was extracted (because β-actin was susceptible to formalin treat-

ment we were not able to utilize β-actin expression for quantitation). The values derived from the metastatic lesion were normalized against the value derived from the primary lesion. Results show that A₃AR is expressed in both primary and metastases of colon and breast (Fig. 3B and Fig. 4B). Moreover, in the colon tissue, A₃AR expression was higher ($P = 0.03$) in the lymph-node metastases than in the primary tumor tissue. In the regional lymph nodes of breast metastases, A₃AR expression was higher ($P = 0.02$) than the relevant primary tumor. (Fig. 3C and 4C).

Comparison between A₃AR mRNA Expression Level in Colon versus Adjacent Normal Relevant Tissues. A₃AR mRNA expression level in six samples of colon carcinoma tissue was examined by RT-PCR analysis and was found to be higher than in the relevant normal tissue (Fig. 5A). To quantitate A₃AR mRNA expression, the density (Et-Br) of the bands related to A₃AR were normalized against that of β-actin (Fig. 5B).

Comparison between A₃AR Protein Expression Level in Colon and Breast Tumor versus Adjacent Normal Relevant Tissues. A₃AR protein expression level in colon tumor tissue was higher in 61% of the specimens in comparison with adjacent normal tissue, whereas in 25.7% of the specimens the

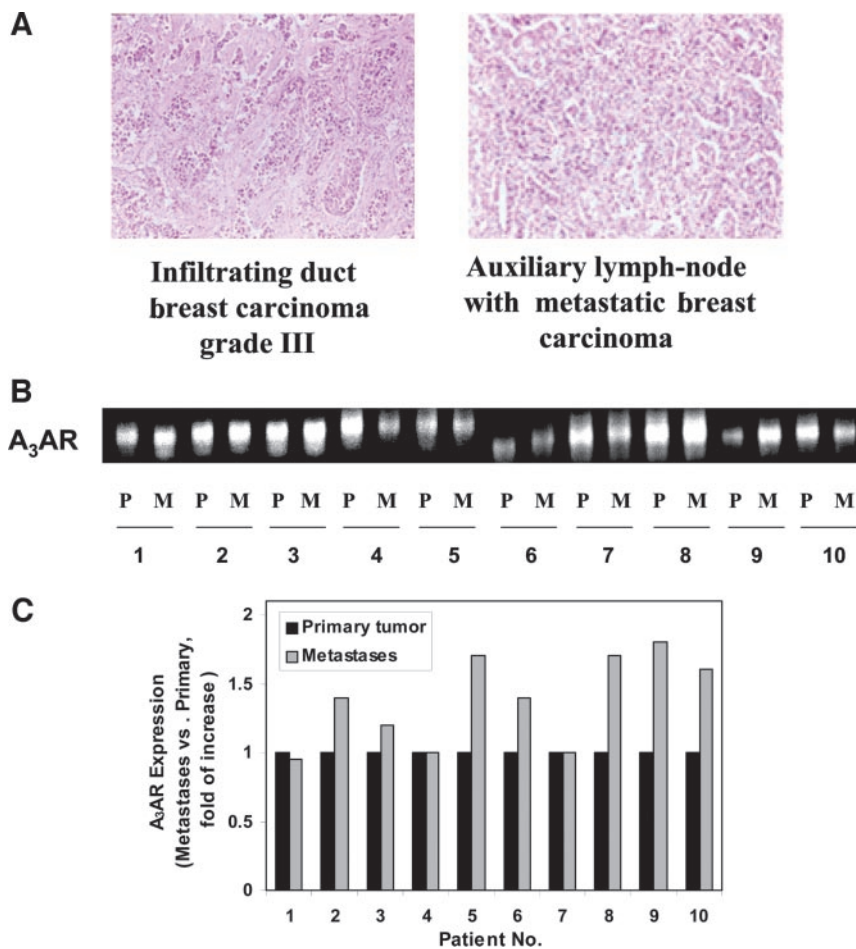


Fig. 4 A₃ adenosine receptor (A₃AR) mRNA expression in primary and metastases of breast carcinoma. *A*, slides mounted with primary and metastatic tissues were marked for neoplastic areas. *B*, mRNA was extracted from the sequential slides and used for reverse transcription-PCR analysis. *C*, quantitation of A₃AR mRNA expression based on the density (ethidium bromide) of A₃AR expression divided by the area from which mRNA was extracted. The values derived from the metastatic lesion were normalized against the value derived from the primary lesion. *P*, primary tumor; *M*, metastasis.

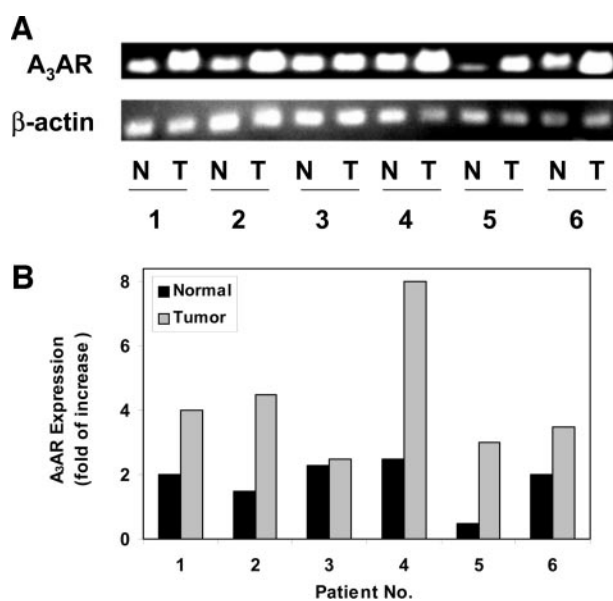


Fig. 5 *A*, A₃ adenosine receptor (A₃AR) mRNA expression in fresh tumor *versus* normal adjacent tissue assayed by reverse transcription-PCR. *B*, quantitation of A₃AR mRNA expression based on β-actin expression in the tissue samples. *N*, normal; *T*, tumor.

expression was lower, and in 13.3% similar A₃AR expression level was observed. Similarly, analysis of the breast tumor tissue revealed that in 78% of the specimens the expression was higher, in 5.3% of the specimens the expression was lower, and in 16.7% the expression was the same as in the adjacent normal tissue (Table 3).

A₃AR, Cyclin D1 and NFκB Protein Expression Level in Colon and Breast Tumor *versus* Adjacent Normal Tissue.

In this experiment we compared the expression level of A₃AR to that of cyclin D1 and NFκB, known to be overexpressed in tumor tissue but low in the relevant normal one. Fig. 6 depicts high expression of the three proteins in the tumor tissue *versus* low in the normal.

A₃AR mRNA Expression Profile in Various Tumor Tissues Examined by RT-PCR.

In an additional set of experiments we examined A₃AR mRNA expression profile in paraffin-embedded slides of small cell lung and pancreas carcinoma, as well as in melanoma. Table 4 depicts a higher score of A₃AR expression in the all of the different tumor *versus* normal tissues.

DISCUSSION

The present study shows that a high A₃AR mRNA expression level is found in colon and breast tumor tissues in compar-

Table 3 Summary of A₃AR^a protein expression level in tumor versus the relevant normal adjacent tissue

The optical density of the bands was quantified using an image analysis system and corrected by the optical density of the corresponding β-actin bands.

	N	A ₃ AR expression (% of incidence)		
		A ₃ AR in tumor		
		>Adjacent	=Adjacent	<Adjacent
Colon carcinoma	40	61.0	13.3	25.7
Breast carcinoma	17	78.0	5.3	16.7

^a A₃AR, A₃ adenosine receptor.

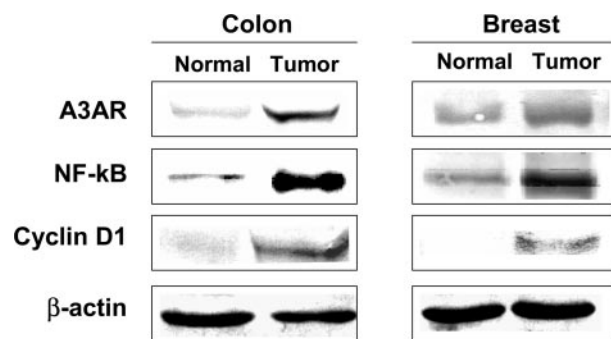


Fig. 6 Expression of A₃ adenosine receptor (A₃AR), nuclear factor κB (NF-κB), and cyclin D1 protein expression level in colon and breast carcinoma versus adjacent normal tissues.

ison with the normal adjacent and normal relevant tissue derived from healthy subjects. Remarkably, a higher mRNA expression level was detected in the regional lymph node metastases in comparison with the primary tumor tissue. In addition, a high A₃AR mRNA receptor level was also detected in other solid tumors including melanoma, colon, breast, renal, ovarian, small cell lung, and prostate carcinoma.

Low A₃AR mRNA expression level was reported earlier as a general characteristic of various normal cell types (13), whereas in tumor cell lines such as melanoma, lymphoma, pineal gland, colon, and prostate carcinoma prominent receptor level was recorded (3–5, 9–12). Dixon *et al.* (14) detected A₃AR message only in the testis tissue using *in situ* hybridization, but found widespread distribution after amplification of the message using PCR. Carre *et al.* (15) examined A₃AR expression in nonpigmented ciliary epithelial cells and found that to establish identity of the A₃AR message, two rounds of PCR amplifications were needed, suggesting that the message is present in low copy number. Atkinson *et al.* (16) studied expression of A₃AR by Northern blot analysis in 35 different human normal tissues. This study revealed that hA₃AR is widely expressed at low to moderate levels. Most abundant levels were found in a number of discrete loci in the central nervous system with low expression in spleen and small intestine.

The present study is the first to compare side by side A₃AR expression level in tumor versus normal tissue, demonstrating that the message is higher in the malignant tissue. A support for this finding came from a search that we conducted in different sources of database, showing a 2.3-fold increase in the expression of A₃AR in human colon adenoma versus normal colon tissue using microarray analysis (Princeton University database). A search in the CGAP (The Cancer Genome Anatomy project; SAGE Genie; Virtual Northern Legend) based on serial analysis of gene expression revealed that A₃AR was abundant in brain, kidney, lung, germ cells, placenta, and retina but brain, lung, and pancreatic tumors expressed more A₃AR in the malignant than the normal relevant tissues. A search in Expression Viewer (HUGO-Gene Nomenclature Committee/CleanEX) based on expressed sequence tags revealed that the relative expression for A₃AR was 1.6-fold higher in all of the cancer tissues compared with normal tissues. A summary of A₃AR expression from the various database searches is presented in Fig. 7.

Table 4 Summary of A₃AR^a mRNA expression level in pathological slides from different tumor tissues

Tissue	Type	A ₃ AR + (n)	Score ^b				Mean score
			1	2	3	4	
Colon carcinoma	Normal	3 (10)	1		2		0.7
	Primary tumor	7 (7)	2	1	2	2	1.7
	Metastases	7 (7)			3	4	2.6
Breast carcinoma	Normal	3 (10)	1			2	0.8
	Primary tumor	10 (10)		5	2	3	1.8
	Metastases	10 (10)		4	3	3	1.9
Small cell lung carcinoma	Normal	0 (1)					0.0
	Primary tumor	1 (1)				1	3.0
Pancreatic carcinoma	Normal	1 (1)	1				1
	Primary tumor	1 (1)			1		3
Melanoma	Normal	2 (3)		2			1.3
	Primary tumor	3 (3)		1	2		2.6

^a A₃AR, A₃ adenosine receptor.

^b Formalin-fixed, paraffin-embedded slides were marked for the neoplastic and normal adjacent tissue. Analysis was carried out on tissue mRNA extracts derived from the unstained sequential slides. Score (1, 2, 3, or 4) for A₃AR mRNA expression levels was determined based on the density values (ethidium bromide) of A₃AR expression in each specimen divided by the cell number. The mean score is the sum of the frequency of each specimen multiply by its respective score, divided by the total number of specimens.

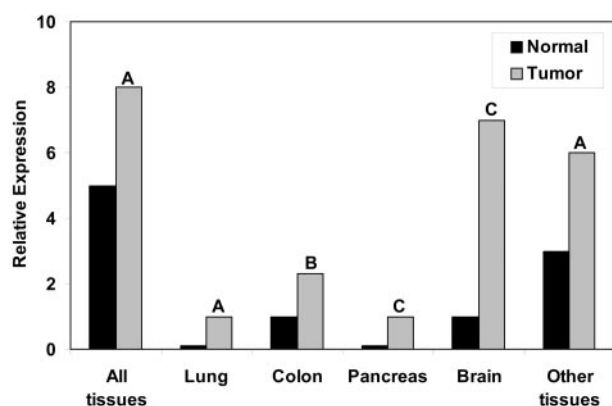


Fig. 7 A₃ adenosine receptor expression in tumor *versus* normal relevant tissue based on (A) expressed sequence tags (expression viewer, HUGO-Gene Nomenclature Committee/CleanEx); (B) microarray analysis, Princeton University database; (C) serial analysis of gene expression, the Cancer Genome Anatomy Project.

A support for the above conclusion that A₃AR is highly expressed in the tumor *versus* adjacent normal tissue was obtained from the analysis of fresh tumors of colon and breast carcinoma. Protein analysis revealed that in >60% of the samples, higher A₃AR expression was found in the tumor *versus* adjacent normal tissue. This was additionally strengthened by similar data that was obtained by mRNA analysis of the colon carcinoma specimens.

In the tumor samples, high A₃AR levels were coexpressed with high cyclin D1 and NFκB levels, whereas the opposite situation was found with the normal relevant adjacent tissue. The level of cyclin D1 and NFκB was found to be down-regulated on A₃AR activation in various tumor cell types (melanoma, prostate, and colon carcinoma) (3, 5) suggesting this highly expressed receptor as a target for tumor growth inhibition.

Interestingly, tumor and normal cells respond differentially to activation of A₃AR by a synthetic agonist. Inhibition of tumor cell growth both *in vitro* and *in vivo* was observed in melanoma, colon, and prostate carcinoma (2–6). On the other hand, the proliferation of normal cells such as murine or human bone marrow was stimulated on cell activation with an A₃AR agonist (7, 8). This differential effect may be explained by the high *versus* low A₃AR expression level in tumor and normal cells, respectively.

The association between A₃AR expression level and functionality was discussed earlier. Black *et al.* (17) transfected the A₃AR gene in cardiomyocytes and tested the effect of gene dosage on protection against ischemia. Interestingly, gene overexpression reversed the protective effect demonstrating that the level of receptor expression plays a role in determining cell response to receptor activation. Dougherty *et al.* (18) found that increased expression of the A₃AR adenosine receptor in cardiac myocytes caused an enhanced cardioprotective effect by improving the myocyte sensitivity to the endogenous adenosine, which, in turn, induces the protective effect. Dhalla *et al.* (19) suggested that an agonist is more efficacious or potent where the

receptor number is high. Thus, receptor expression is cell type specific and reflects the response to a given agonist.

The high A₃AR expression in neoplastic cells may be attributed to high adenosine level in the microenvironment of the tumor, released by necrotic or hypoxic cells. During homeostasis, the physiological levels of adenosine do not reach the concentrations needed to activate A₃AR. A₃AR has the lowest affinity to the natural ligand adenosine, which is ~1 μM (1). Therefore, it may be suggested that the elevation in the extracellular adenosine concentration may trigger more receptor expression by the tumor cells. In addition, it may happen that in tumor cells, overexpression of transcription factors, responsible for A₃AR expression, takes place, resulting in up-regulation of receptor mRNA and protein levels. In previous studies we took advantage of receptor overexpression and targeted the receptor with a synthetic agonist, which induced tumor growth inhibition.

To conclude, high mRNA and protein A₃AR expression level was detected in various tumor cell types, classifying this G_i protein receptor to the family of other receptors, such as the epidermal growth factor, and suggesting it as both diagnostic and therapeutic target.

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