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Characterization of the cell membrane during cancer transformation

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Abstract: *The electric properties of the plasma membrane is an indicator of cell condition. The simple, and highly effective, normal-phase (NP) and reversed-phase (RP) high-performance liquid chromatography (HPLC) methods assess phospholipid and free unsaturated fatty acid content, respectively. Herein we focus on changes in phospholipid content [phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanoloamine (PE), phosphatidylcholine (PC)] and free unsaturated fatty acid content [arachidonic acid (AA), linoleic acid (LA), α-linolenic acid (ALA), palmitoleic acid (PA)] in the plasma membranes of non-metastatic colorectal cancer cells (pT3 stage, G2 grade). Surface charge density of normal and tumor large intestine tissue was measured by electrophoresis. The surface charge density as a function of pH, acidic (C_{TA}) and basic (C_{TB}) functional group concentrations and their average association constants with hydrogen (K_{AH}) or hydroxyl (K_{BOH}) ions were evaluated. Cancer transformation was accompanied by an increase in total phospholipids as well as and increase in C_{TA}, C_{TB} and K_{BOH} whereas the content of free fatty acids and K_{AH} decreased compared with unchanged tumor cells.*

Key words: *Phospholipids, Free fatty acids, HPLC, Electric charge, Electrophoresis, Colorectal cancer cells*
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Introduction

Biological membranes are essential boundaries within the living cell. Plasma membranes separate the interior of the cell from the environment and participate in intercellular communication. The most important property of a biological membrane is its electrical charge and the difference in potential drop between the membrane and the surrounding solution. Cell membrane charge increases during tumorigenesis and decreases during necrosis (Dolowy, 1984). Determining the electric charge of the membrane as function environmental pH, acidic (C_{TA}) and basic (C_{TB}) functional group concentrations and their average association constants with hydrogen (K_{AH}) or hydroxyl (K_{BOH}) ions allows researches to monitor the changes caused by cancer transformation (Dobrzynska *et al.*, 2006).

The electrical properties of the membrane are determined by acid-base and complex formation equilibria in which membrane and surrounding medium components are involved (Gennis, 1989; Tien, 1974). Most membrane components, including proteins, phospholipids and fatty acids participate in those interactions. Membrane phospholipid composition and free fatty acid composition are modified under many different conditions such as cancer transformation (Dobrzynska *et al.*, 2005; Szachowicz-Petelska *et al.*, 2007). The modifications are extensive enough to alter membrane fluidity and affect a number of cellular functions, including the properties of certain membrane-bound enzymes, immune cell activation (both cell proliferation and phagocytosis) and tumor growth. The changes result in increased de novo synthesis and membrane phospholipid turnover (Field and Schley, 2004).

Examining the electrical charge could reveal substantial information about the balance between membrane components but also between membrane components and the surrounding solution. The electrical charge is determined by structural positive charge carriers (free amino groups of proteins and aminophospholipids) and by negative charge carriers [some phospholipids (especially phosphatidylserine), sialic acid, glycophorines and free carboxy groups of polypeptide chains]. Therefore, estimating the quantity of membrane components by analytical methods and taking advantage of high performance liquid chromatography (HPLC) as well as estimating charge by electrophoresis, should lead to compatible results.

Previously, we published data characterizing changes in membrane phospholipids as a result of exogenous factors (Dobrzynska *et al.*, 2006). Therein, we hypothesized on the relationship between the biochemical changes and the exogenous factors. Endogenous factors are also important and can be discussed in a similar way. In this work, we examine a particular factor, transformation, and the changes in membrane composition and electrical properties.

Materials and Methods

Tissue samples were obtained from six patients (2 men and 4 women) who underwent surgical resection for colorectal cancer. The patients ages ranged from 45 to 80 years old. The tumors were grade G2 and stage pT3 without distant and lymph

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node metastases, classified histopathologically as adenocarcinoma. Tumor samples and normal colon mucosa were collected immediately after tumor removal. The segments from macroscopically disease free intestinal mucosa were taken at a distance not smaller than 10 cm from the neoplastic lesions.

Isolation of cell membrane: Tissues (approximately 1.0 g) were homogenized in a solution containing 1 mM-$NaHCO_3$ (pH=7.6) and 0.5 mM $CaCl_2$ in a loose-fitting Dounce homogenizer. Membrane fragments were separated from nuclei and mitochondria by rate-zonal centrifugation of the 'low-speed' pellet as described previously (Evans, 1970). The sediment was homogenized in sucrose (1.22 g cm^{-3} density) and in the next step was covered with sucrose (1.16 g cm^{-3} density). The cell membranes were separated by centrifugation at 2000xg for 25-35 minutes (Dobrzynska *et al.*, 2005).

Analysis of phospholipids: The Folch method was used to extract phospholipids (Szachowicz-Petelska *et al.*, 2002). The cell membrane was homogenized in a chloroform–methanol mixture of (2:1 volume ratio). The solution was then filtered with degreased paper filters, and the precipitate was washed with an extracting solution (8:4:3 chloroform:methanol:aqueous calcium chloride solution 0.05 M calcium chloride). The suspension was centrifuged at 500xg for 2 minutes, the organic and the aqueous phases were separated, and the aqueous phase was shaken again with chloroform, methanol and water mixture of (3:48:47 volume ratio) and the phases were separated. The organic phases were combined and evaporated to dryness. The extract was dissolved in 200 ml of hexane:isopropanol mixture (3:2) (Szachowicz-Petelska *et al.*, 2002). Addition of 0.03% tert-butylhydroxytoluene (BHT) and flushing with nitrogen at each step in the procedure were used to prevent oxidation during lipid extraction.

HPLC analysis was performed on the extracted phospholipids to assess the quantities phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) from normal human large intestine cells. The isolated phospholipids were separated by group analysis in a silica gel column using normal phase (NP)- HPLC; acetonitryle–methanol-phosphoric acid (85%) mixture (130:5:1,5 volume ratio) by isocratic elution at 1 ml sec^{-1} flow rate and 214 nm wave length (Ostrowska *et al.*, 2000).

Analysis of free unsaturated fatty acids: The cell membrane was homogenized in a 2% acetic acid-ethyl ether mixture (2:1 volume ratio). The solution was then filtered with degreased paper filters. The filtered suspensions were centrifuged at 500xg for 2 min, the organic and the aqueous phases were separated, the aqueous phase was shaken again with 2% acetic acid in ethyl ether (2:1 volume ratio) and the phases were separated (Ostrowska *et al.*, 2000). The organic phases were combined and evaporated to dryness. The extract was dissolved in 200 μl acetonitrile (Lim, 1986).

HPLC analysis was then performed on the dissolved extract, a typical chromatographic separation of only free unsaturated fatty acids: arachidonic acid (AA: 20:4n-6), linoleic acid (LA: 18:2n-6), α-linolenic acid (ALA, 18:3n-3), palmitoleic acid (PA: 16:1). The isolated free unsaturated fatty acids were separated by group analysis in an RP 18 column using reverse phase (RP)-HPLC using an acetonitrile-water mixture (70:30 volume ratio) by isocratic elution with a 1 ml s^{-1} flow rate and 214 nm wavelength, as desribed previously (Aveldano *et al.*, 1983).

Electrochemical method: In order to determine surface charge density of cell membrane, colorectal cancer tissue from human was exposed to trypsin action. Received cells were put into the measuring vessel, then electrophoretic mobility on dependent pH was measured by using DTS5300 ZETASIZER 3000 apparatus (MALVERN INSTRUMENTS).

The surface charge density has been determined using equation: $\delta=\eta u/d$; where u is the electrophoretic mobility, η in the viscosity of the solution and d is the diffuse layer thickness (Krysisski and Tien, 1986). The diffuse layer thickness was determined from the formula (Barrow,1996) $d=\sqrt{\frac{\varepsilon\cdot\varepsilon_0\cdot R\cdot T}{2\cdot F^2\cdot I}}$, where R is the gas constant, T is the temperature, F is the Faraday number, I is the ionic strength of 0.9 % NaCl, and ε and ε_o – are the relative and absolute permittivities of the medium.

Acidic (C_{TA}) and basic (C_{TB}) functional group concentrations and their average association constants with hydrogen (K_{AH}) or hydroxyl (K_{BOH}) ions were determined as described previously (Dobrzynska *et al.*, 2006).

Statistical methods: All statistical analyses were performed with the standard statistical program SPSS 8.0 PL. The data obtained in this study are expressed as the mean ± the standard deviation (SD). The data were analysed using the Wilcoxon Matched-Pairs Signed-Ranks Test to compare control and cancer samples. P value of less than 0.05 was considered significant.

Results and Discussion

The phospholipid content in human large intestine cell membranes and in the membranes of cells from the neoplastic lesion are presented in Table 1. Generally, an increase in the content of all phospholipids is observed in the neoplastic lesions compared to the unaffected cells. An increase in the amount of phospholipids is generally observed in human colon cancer (Dueck *et al.*, 1996) and in murine mammary tumor cells (Monteggia *et al.*, 2000). Increased phospholipid content may result from enhanced cell membrane synthesis related to accelerated neoplasm cell replication (Ruiz-Cabello and Cohen, 1992). The mechanisms responsible for increased phospholipid content can vary depending on cell type, cell growth phase and malignancy. The greatest changes in PC and PE content were observed in the G_1 phase of the cell cycle, in which activity of the enzymes controlling biosynthesis, catabolism and metabolism of phospholipids is the highest (Jackowski, 1996; Jackowski, 1994). The data presented in Table 2 also show that PC content in normal mucosa or lesions of colorectal cancer cells is

Table - 1: Phospholipid content of human large intestine cell membranes

Patient No's	Phospholipids	Content of phospholipids of plasmalemma (mg g^{-1} tissue)	
		Control	Tumour
1	PI	0.010 ± 0.003	0.109 ± 0.040[a]
	PS	0.030 ± 0.002	0.765 ± 0.049[a]
	PE	0.410 ± 0.020	0.151 ± 0.022[a]
	PC	0.576 ± 0.010	0.640 ± 0.051[a]
2	PI	0.077 ± 0.009	0.198 ± 0.020[a]
	PS	0.089 ± 0.007	0.099 ± 0.006[a]
	PE	0.932 ± 0.050	0.999 ± 0.060[a]
	PC	1.113 ± 0.061	1.224 ± 0.089[a]
3	PI	0.054 ± 0.005	0.099 ± 0.010[a]
	PS	0.081 ± 0.004	0.114 ± 0.015[a]
	PE	0.398 ± 0.012	0.694 ± 0.080[a]
	PC	0.577 ± 0.018	0.943 ± 0.095[a]
4	PI	0.028 ± 0.002	0.063 ± 0.006[a]
	PS	0.030 ± 0.002	0.081 ± 0.009[a]
	PE	0.489 ± 0.012	0.833 ± 0.092[a]
	PC	0.684 ± 0.019	1.201 ± 0.101[a]
5	PI	0.010 ± 0.001	0.028 ± 0.015[a]
	PS	0.015 ± 0.002	0.025 ± 0.010[a]
	PE	0.385 ± 0.023	0.748 ± 0.052[a]
	PC	0.545 ± 0.034	1.018 ± 0.065[a]
6	PI	0.035 ± 0.003	0.087 ± 0.040[a]
	PS	0.042 ± 0.002	0.097 ± 0.022[a]
	PE	0.457 ± 0.020	0.740 ± 0.061[a]
	PC	0.589 ± 0.010	0.887 ± 0.059[a]

PI-phosphatidylinositol; PS-phosphatidylserine; PE-phosphatidylethanoloamine; PC-phosphatidylcholine. Statistically significant differences for $p<0.05$. [a]In comparison with control

Table - 2: Free unsaturated fatty acids content of human large intestine cell membranes

Patient No's	Fatty acids	Content of fatty acids of plasmalemma (mg g^{-1} tissue)	
		Control	Tumour
1	18:2n-6	0.039 ± 0.004	0.029 ± 0.0035[a]
	18:3n-3	0.042 ± 0.003	0.032 ± 0.0035[a]
	16:1	0.029 ± 0.003	0.027 ± 0.007
	20:4n-6	0.041 ± 0.004	0.052 ± 0.003[a]
2	18:2n-6	0.030 ± 0.004	0.019 ± 0.0035[a]
	18:3n-3	0.066 ± 0.003	0.051 ± 0.0035[a]
	16:1	0.025 ± 0.003	0.028 ± 0.0025
	20:4n-6	0.054 ± 0.004	0.066 ± 0.003[a]
3	18:2n-6	0.030 ± 0.004	0.019 ± 0.0025[a]
	18:3n-3	0.045 ± 0.003	0.029 ± 0.0025[a]
	16:1	0.024 ± 0.003	0.025 ± 0.0025
	20:4n-6	0.048 ± 0.004	0.062 ± 0.003[a]
4	18:2n-6	0.022 ± 0.002	0.013 ± 0.0015[a]
	18:3n-3	0.024 ± 0.001	0.018 ± 0.001[a]
	16:1	0.018 ± 0.001	0.019 ± 0.002
	20:4n-6	0.028 ± 0.002	0.031 ± 0.0025[a]
5	18:2n-6	0.018 ± 0.002	0.010 ± 0.0015[a]
	18:3n-3	0.029 ± 0.002	0.021 ± 0.0025[a]
	16:1	0.018 ± 0.002	0.020 ± 0.002
	20:4n-6	0.035 ± 0.002	0.041 ± 0.002[a]
6	18:2n-6	0.015 ± 0.002	0.008 ± 0.0025[a]
	18:3n-3	0.030 ± 0.001	0.018 ± 0.0015[a]
	16:1	0.009 ± 0.001	0.010 ± 0.002
	20:4n-6	0.024 ± 0.002	0.036 ± 0.002[a]

18:2n-6, linoleic acid; 18:3n-3, a-linolenic acid; 16:1, palmitoleic acid; 20:4n-6, arachidonic acid, Statistically significant differences for $p<0.05$. [a]In comparison with control

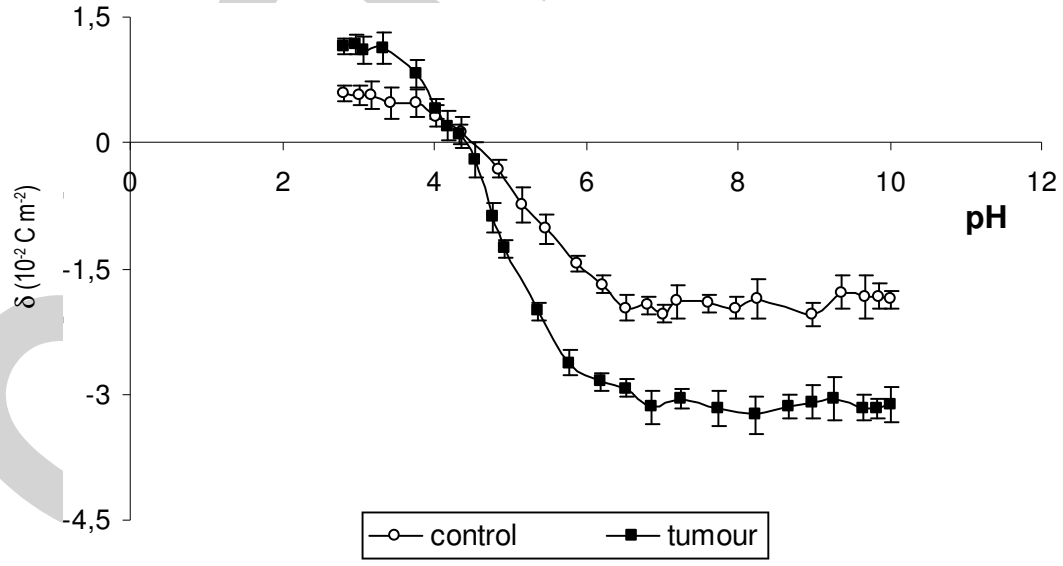

Fig. 1: Dependence on pH of surface charge density of normal and tumor large intestine cells from several patients

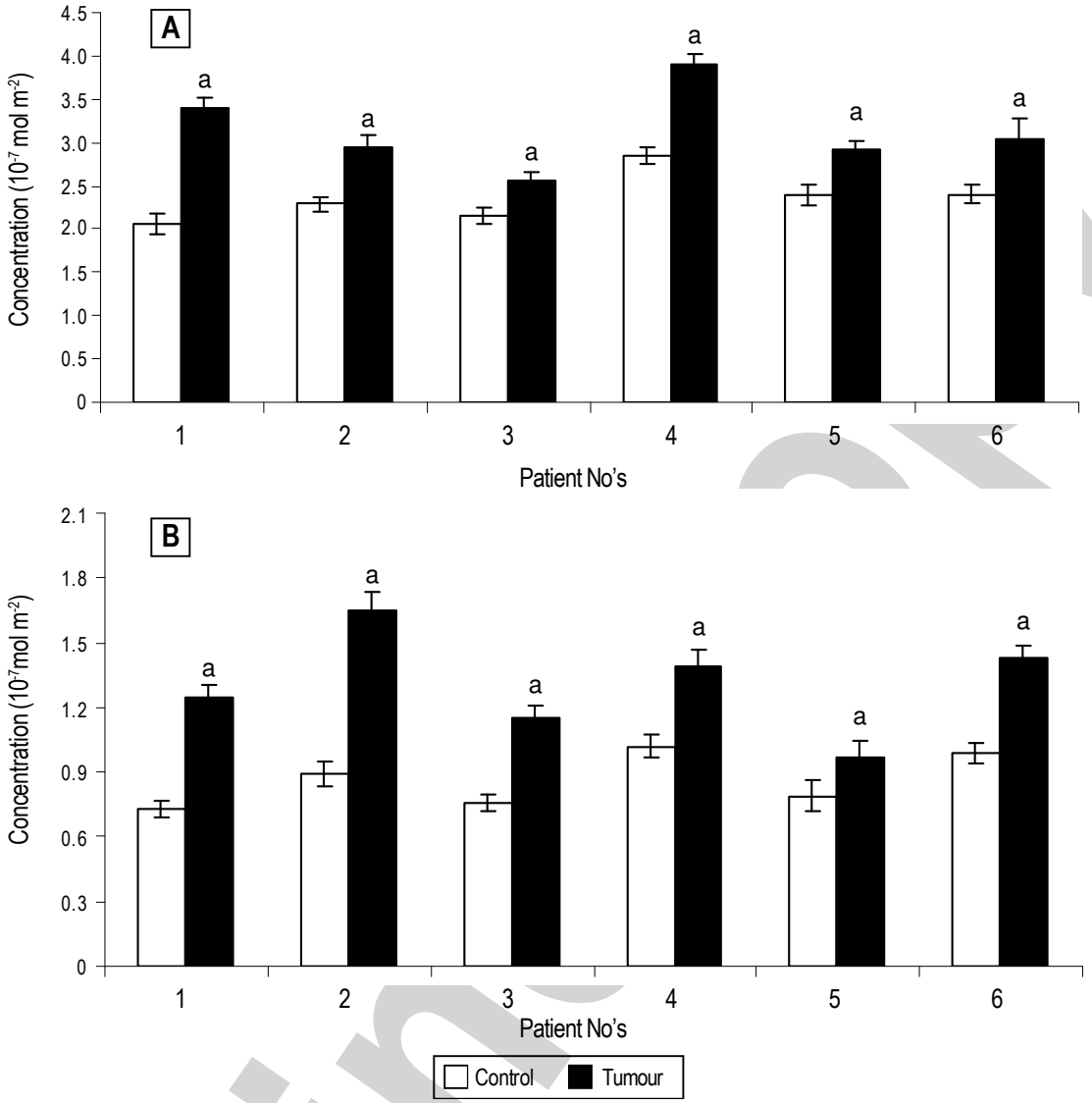

Fig. 2: The acidic functional group concentrations (A) and basic functional group concentrations (B) of human large intestine cell membranes

higher than other phospholipids. PC content is higher still in the cancer cells compared to the normal mucosal cells confirming earlier reports (Dueck *et al.*, 1996).

The LA and ALA content decreased significantly and the AA content increased significantly in the tumor cells compared to unaffected cells (Table 2). The change in AA content was more significant than changes in the other fatty acids, both in control and in cancer tissues. Generally, the content of all free fatty acids decreased. Decreased LA and ALA were reported in the plasma and in erythrocytes of colorectal cancer patients (Baro *et al.*, 1998; Fernandez-Banares *et al.*, 1996). These changes are probably due to a metabolic alteration caused by the illness but not by malnutrition (Baro *et al.*, 1998). Two clinical investigations reported a significant increase in plasma and tissue AA concentrations in colorectal cancer patients compared with controls (Neoptolemos *et al.*, 1991; Hendrickse *et al.*, 1994) which could be the result of enhanced lipid peroxidation, a feature of rapidly growing cells (Skrzydlewska *et al.*, 2001; Skrzydlewska *et al.*, 2005).

Alternatively, the increased AA levels could be due to elevated desaturase activity on LA and ALA leading to increased formation of prostaglandins and other lipoxygenase products (Dommels *et al.*, 2002).

Surface charge density dependences on pH of normal and tumor large intestine cell membrane are similarly shaped (Fig. 1). There is an increase in positive surface charge density of the cells at low pH values until a plateau is reached. At high pH values, the negative charge of the cells also increases reaching a plateau. Overall, an increase in negative charge at low pH values as well as in positive charge at high ones is observed in human large intestine tumor cells compared to unaffected cells. The C_{TA}, C_{TB} and K_{BOH}

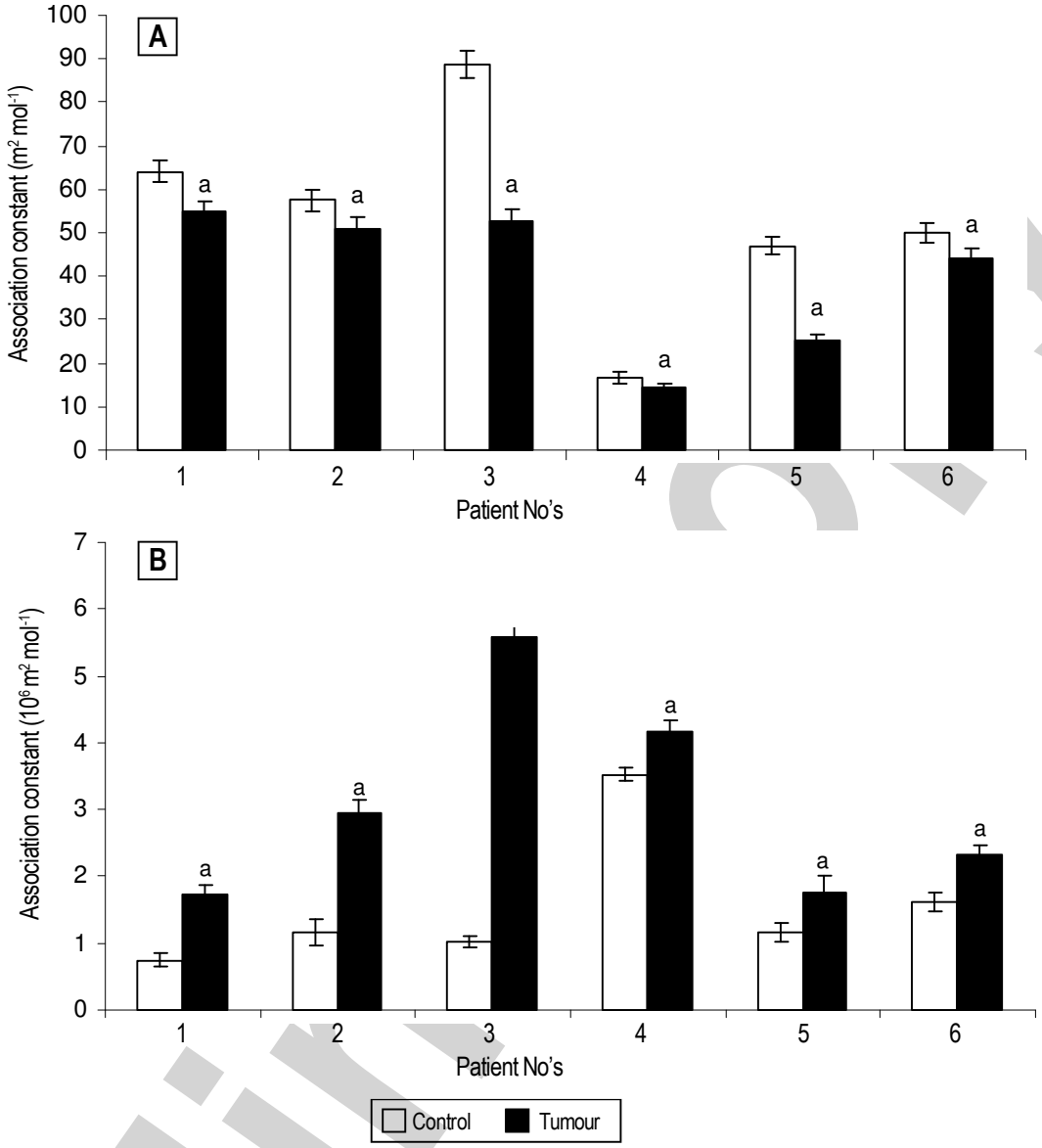

Fig. 3: The average association constant with hydrogen ions (A) and hydroxyl ions (B) of human large intestine cell membranes

values of the cell membranes modified by cancer transformation were higher than in unmodified cells (Fig. 2 and 3) while K_{AH} was decreased in comparison (Fig. 3).

Enhanced phospholipid content results in a higher number of functional amino, carboxy and phosphate groups. In acidic medium (low pH), phospholipids charge is mainly due to amino groups whereas in basic medium (high pH), it is due to carboxy and phosphate groups. Increased phospholipid levels can increase surface concentrations of acidic group (C_{TA}) and basic group (C_{TB}) of the large intestine cell membrane (Fig. 2). The main component of the large intestine cell membrane outer layer is PC: higher PC content increases C_{TA} and C_{TB} values. Cancerous transformation lowers the association constant of negatively charged groups (K_{AH}) and raises the association constant of positively charged ones (K_{BOH}) (Fig. 3). Anionic phospholipids on tumor vessels could potentially provide markers for tumor vessel targeting and imaging (Ran *et al*., 2002). Alterations in the PS distribution a component of the skeleton, could increase the C_{TA} value.

Beside the phospholipids discussed in this work, cell membrane charge is also affected by sialic acid a component of glycolipids and glycoproteins. Sialic acid also may influence surface concentrations of acidic and basic groups, as well as the association constants of positive and negative groups during cancerous transformation. Increased sialic acid content in glycolipids and glycoproteins has been confirmed (Jakielaszek *et al*., 1986; Erbil *et al*., 1986).

The results obtained here show that cell membrane structure and function are modified in neoplastic lesions, as reflected by

changes in phospholipid and free fatty acid content and in the C_{TA}, C_{TB}, K_{AH}, K_{BOH} parameters of human large intestine cell membranes.

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