INTRODUCTION

Polyps are markedly specific proliferative pathologies of the colon. Causes of their formation, growth rate, and tendency to recur remain unknown. It is widely known that a small percentage of adenomatous polyps can turn into invasive carcinoma; however, reliable methods to select the patient group have not been yet developed. According to Jacobson-Brown and Neuman (1) the growth factors, oncogenic cytokines (IL-2, IL-12, IFN-gamma, TNF-alpha, and TRAIL) and immunosuppressive factors may play a crucial role in the growth and survival of premalignant colonic tissue. The progression from precancerous (adenomatous) colon polyps to malignant colorectal cancer involves the complex actions of various cytokines on T cell proliferation, cell-cell adhesion, apoptosis and host immunity (2). There are single reports concerning the role of selected toll-like receptors (TLRs) in colonic pathologies; they are believed to induce apoptotic cell death of neoplastic cells.

TLRs constitute a kind of link between innate (non-specific) and adaptive (specific) immunity, contributing to the capacity of our immune system to efficiently combat pathogens. They also enable immune cells to discriminate between self and non-self antigens (3). It is innate immunity that helps...
recognize and react against a particular molecular structure of bacteria, viruses, protozoans and fungi. TLR expression was observed in immune system cells, epithelium, vascular endothelium, adipocytes, cardiomyocytes, fibroblasts, and keratinocytes. They are mainly expressed on cell membrane (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11) although some have also been detected in cytoplasmic vesicle membrane (TLR3, TLR7, TLR8, TLR9) (4).

The major role of TLR receptors in colonic and respiratory epithelium, endothelial cells (TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR9) and adipocytes is prompt recognition of infectious pathogens and triggering a mechanism responsible for their eradication. Activated epithelial cells release considerable quantities of proinflammatory cytokines, chemokines and defensins, which, in turn, alert immune system cells (leukocytes, macrophages, mast cells, and dendritic cells).

Our knowledge on the role of TLR receptors in polyp formation, recurrence after removal, growth rate, and possible malignant transformation is quite limited. The studies of Jacobson-Brown and Neuman revealed that polyp to cancer transformation stimulates the immune system resulting in IL-2, IL-12, INF-gamma and TNF-alfa expression, which exhibit cytotoxic and apoptotic effects.

An interesting question is which TLR receptors are over- and which underexpressed in polyposis, and what are mutual relationships thereof. Also, whether it would be possible, based on TLR gene expression, to predict malignant polyp potential, and evaluate the tendency of normal tissue to form polyps or undergo malignant transformation.

The purpose of the present study was to identify differences in TLR gene expression patterns in normal and diseased tissues of patients with polyps and colorectal cancer as well as try to find the solution to the above-mentioned dilemmas.

MATERIAL AND METHODS

Eight patients were included in the study group (2 women and 6 men aged 38 to 72 years).

Pathologic tissue specimens were collected during colonoscopy (apical region of the polyp, surgical margin), and, in each case, macroscopically normal colon mucosa 8 centimeters from the lesion. Sixteen HG-U133A oligonucleotide microarrays were analysed including four of colon polyps, four of adenocarcinoma with different degree of histological differentiation (2 poorly and 2 highly differentiated), and eight of macroscopically normal tissue from 4 polyposis and 4 adenocarcinoma patients (Fig. 1). The levels of selected TLR mRNA transcripts were analyzed.

A comparative analysis was carried out of the transcriptomes of polyp, adenocarcinoma and normal tissue specimens using human genome microarray platforms, the Affymetrix HG-U133A for estimation of all genes that will be described in the next manuscript (5). Molecular analysis consisted of three stages.

Following mRNA hybridization, the copy number of mRNA in colonic specimens was 22284. Using Microarray Analysis Suite, reports were generated which were used to evaluate the obtained results and include transcriptomes in the comparative analysis. RMA Express was used to normalize background and noise, and thus diminish the probability of result misinterpretation. Afterwards, based on the measures of descriptive statistics, microarray plots, and Spearman’s rank correlation coefficient, the quality of results was again evaluated to confirm transcriptome inclusion in the analysis.

The third stage consisted of differentiation between normal and pathologic tissue transcripts. Subsequently, attempts were undertaken to identify relationships between selected TLR mRNA transcripts and colon pathologies compared to normal colonic specimens.

RNA extraction

RNA from polyp and normal tissue specimens was isolated with TRIzol reagents (Gibco) following manufacturer’s protocol. RNA samples were then treated with DNase I and prepared with the RN easy Mini Kit by Qiagen column kit. RNA quantity was determined by absorbance at 260 nm using a spectrophotometer and its quality was evaluated by electrophoresis on 2% agarose gel. The obtained extract was used to analyse cell transcriptomes by oligonucleotide microarrays (5).
Oligonucleotide microarray transcriptome analysis

Oligonucleotide microarray transcriptome analysis started with cDNA synthesis from RNA extract; 1µl of 100 pM starter T7-oligo (dT)$_{24}$ (5'-GCCAGTGAATTGATAACGACTCACTATAGG GAGGCGG-3') was added to 8 µg RNA and incubated at 70°C for 10 minutes. The following were then added to cDNA synthesis mix: 4 µl 5x First Strand Buffer, 2 µl 0.1 M DTT and 1 µl 10 mM dNTPs. Following a 2-minute incubation at 42°C, 2 µl (200 U) of reverse transcriptase Superscript II (Life Technologies) were added to the mixture and incubated for one hour. In order to produce a complementary cDNA strand, 30 µl 5x Second Strand Buffer, 91 µl of RNase-free water, 3 µl 10 mM dNTPs, 4 µl (40 U) E. coli DNA Polymerase I (Life Technologies), 1 µl (10 U) E. coli DNA Ligase (TaKaRa), and 1 µl (2 U) RNase H (TaKaRa) were subsequently added, and then incubated at 16°C for 2 hours. Afterwards 2.5 µl (10 U) T4 DNA Polymerase I (TaKaRa) were added and incubated at 16°C for 5 more minutes. The reaction was stopped by adding 10 µl 0.5 M EDTA to the reaction mix, and double-stranded cDNA was extracted with phenol/chloroform. The aqueous layer was isolated using Phase Lock Gel (Eppendorf). cDNA precipitate was dissolved in 12 µl RNase-free water. 10 µl of double-stranded cDNA was used for biotin-labeled cRNA synthesis (BioArray High Yield RNA Transcript Labeling Kit, Enzo Diagnostic). Biotinylated cRNA was purified in mini spin columns of the RNeasy Mini Kit by Qiagen, fragmented and hybridised – first with test microarray (Test 3), and subsequently using microarray platform Human Genome Arrays U133A (Affymetrix). Washing, staining by streptavidin-phycoerythrin, and microarray scanning in GeneArray (Agilent) were performed according to Affymetrix Gene Expression Analysis Technical Manual.

Microarray data analysis using modern data base resources

Result analysis was performed in the Institute of Computer Science of the University of Silesia. Input data of particular tissue groups was evaluated. The purpose of the analysis was to assess the homogeneity of the obtained fluorescence values of the same transcripts within different groups. Evaluation parameters may serve as measures of both homogeneity (low value of the parameter) or heterogeneity (variability).

Readings variability was defined by a statistic measure referred to as variance. Variance was calculated for all transcripts of each group (normal, polyp, adenocarcinoma in different stages). The analysis required that high and low variance should correspond to high and low readings variability, respectively. So, basing on variance value we can state if expression level readings are “more or less” of the same value. Low variance means low expression levels differentiation – in this case we can compute mean and standard deviation - has sense. When variance - ease of readings variability has high value - eadings in groups are different: higher variance means less sense to state that mean is rational as representing value. That is why has been decided do not use mean and standard deviation. This could be a some kind of falsification of the data. Moreover - the quantity of each examined group was too small to justify full and classical statistical approach. Instead, it has been used min and max values in each examined group, plus variance - as measure of data variability. And, there has been decided to use a relatively new approach to advanced data analysis - exploratory data analysis (18). Generally, in this approach, considered often as a part of data mining (or knowledge discovery from data), many of data analysis methods and techniques can be used - but most techniques are based on graphical representation of the data. So, in this work we used data visualisation – and there has been written what can be noticed in the examined data “at first sight”. Variances has been marked as var. Minimum and maximum values in each examined group are marked as minGroup_symbol, maxGroupSymbol. The highest and lowest values of the readings were also noted as colors of the table cells. Then, taking into account variability of reading in each groups (variance of readings), changes in transcripts expression levels has been calculated. Change values has been encoded as table cells colors (green - decreased expression, red - increased expression). With such data visualisation it is easy noticeable (see Table 1) that for example, for TLR 2 transcript expression level are changing - from lower in polyps tu higher - in adenocarcinoma stage 4 - this can be interpreted as growing activity of TLR 2, and can suggest to continue further investigation to check out if TLR2 could potantially be considered as a marker of adenocarcinoma stage.

Next, there has been checked out for which TLRs expression levels are similar or different for polyps and normal colon mucosa (Fig. 2) and for highly and lowly differentiated adenocarcinoma (Fig. 3). Because we used min-max values of the each group, not mean with standard deviation, we are able to see all range of variability of expression in polyps (diamonds) and normal colon mucosa (crosses) on Fig 2. Ranges for highly differentiated
adenocarcinoma (diamonds) and lowly differentiated adenocarcinoma (crosses) are shown in a similar way on Fig. 3. Thanks to such data representation, and without insertion additional, “artificial” number we have much wider view on expression variability. Basing on Fig. 2, we can “read” for example that expression values for polyps and normal colon mucosa are in disjunctive ranges for TLR2 and TLR4, so we can move a proposal that those TLR’s can be “candidates” for markers for polyps, TLR8 expression range is almost the same for both groups, so it is probable that this will not be a good marker. In the rest TLR’s ranges are either included one within the second or has a common part – so we can not state any further conclusion without additional data and/or experiments.

RESULTS

The minimum mRNA transcript readings in normal tissue were obtained for TLR5 (2.48), and the maximum for TLR6 (10.25). The highest variance was observed for TLR6 (2.29), and the lowest for TLR5 (0.05). Per cent variability of transcript expression level in polyps and normal colon mucosa was the highest for TLR4 (13.559), and the lowest for TLR6 (-20.823). Per cent variability of transcript expression level in stage I adenocarcinoma and normal colon mucosa was the highest for TLR8 (-1.867), and the lowest for TLR3 (-14.625) whereas in stage III adenocarcinoma and normal colon mucosa per cent variability of transcript expression level was the highest for TLR2 (32.864), and the lowest for TLR3 (-23.913). An analysis of all per cent variability values with regard to malignancy stage increasing from polyp to stages I to III adenocarcinoma, and normal colon mucosa shows a statistically significant relationship for TLR2 (increasing) and TLR3 (decreasing) (Table 1).

In polyps, copy numbers of TLR3, TLR4 and TLR5 mRNA were the highest and TLR7 mRNA the lowest. In normal colon mucosa of polyposis patients the highest mRNA copy numbers were observed for TLR3, and the lowest for TLR7.

In highly differentiated stage I and II adenocarcinoma, mean copy number of mRNA was the highest for TLR3 (4.88), and the lowest for TLR7.
Similar results were obtained in low differentiated stage III and IV adenocarcinoma where the mean copy number of TLR3 mRNA was also the highest (4.99), and of TLR7 mRNA the lowest (3.2) (Table 2).

In normal colonic mucosa of highly and low differentiated adenocarcinoma and polyposis, a significant decrease in per cent variability was only observed for TLR3 transcript expression (-8.81; -10.82) whereas an increase was found for TLR2 (93.45;13.84).

A comparison of minimum and maximum TLR mRNA expression in polyps, adenocarcinoma and normal colon mucosa is presented in Figs 2 and 3.

**Table 1.** mRNA transcript readings for TLR: minimum, maximum, variance in macroscopically normal mucosa (var norm), variability in normal and pathologic tissues: polyp ( variab norm vs polyp), and different stage adenocarcinoma ( variab norm vs St-I-IV). Green (lowest transcript expression) and light green table cells shows decreasing of transcript expression, yellow – insignificant change of expression, orange and red (highest expression change) - increasing transcript expression.

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<th>Gene Symbol</th>
<th>Genename</th>
<th>min norm (polyp)</th>
<th>max norm (polyp)</th>
<th>min norm (poly)</th>
<th>max norm (poly)</th>
<th>TLR 2</th>
<th>TLR 3</th>
<th>TLR 6</th>
<th>TLR 5</th>
<th>TLR 1</th>
<th>TLR 8</th>
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**DISCUSSION**

At present colorectal carcinoma is one of the most significant social and medical problems, and, in different countries, the second or third leading cause of cancer-related death. Two inherited conditions, i.e., familial polyposis and hereditary nonpolyposis colon cancer (HNPPC) increase colon cancer risk. DNA mismatch repair genes hMSH2, hMLH1, hPMS1, hPMS2, hMSH6 and hMSH3 contribute to a susceptibility to the two types of colon cancer; hMSH2 and hMLH1 are assumed to account for 90% of the cases. Previous research indicated that colon adenoma was a precursor to 70-90% of colon cancer.
Adenoma resection, widely recognized as cancer prophylaxis, is not always sufficient. Martinez et al. (6) demonstrated adenoma formation in 50% of their patients at three years of polypectomy. Recurrence of advanced adenoma (of > 1 cm in diameter, with villous element) was associated with the size of primary polyps (> 1 cm) and their location in the colon. The authors also confirmed earlier reports on proximal migration of colorectal neoplasms. Thus, the need for CRC screening with total colonoscopy, adenoma removal, and post-polypectomy surveillance seems well-justified. The role of TLR receptors in colorectal pathology has not been fully elucidated. TLR receptors recognize bacterial pathogens resulting in signaling pathways stimulation and consequent proinflammatory activation through nuclear transcription factor NF(κappa)B. TLR stimulation by microbial metabolites constitutes a signal activating the mechanisms of non-specific immunity. Commensal bacteria normally present in the intestinal microflora can inhibit NF(κappa)B through TLRs stimulation and limitation of the inflammatory process. The role of TLR in non-specific inflammatory response has been widely investigated. Inflammation is believed to affect cell proliferation as well as apoptosis. However, although a lot of data are available on TLR4 expression in non-specific intestinal inflammation (the receptors can bind LPS, a structural component of outer membranes of Gram-negative bacteria), little is known about TLR5 expression changes in ulcerative colitis and Lesniowski-Crohn disease. The receptor may play a significant role in the initiation and sustaining the inflammatory process through TLRs stimulation and limitation of the inflammatory process. The role of TLR in non-specific inflammatory response has been widely investigated. Inflammation is believed to affect cell proliferation as well as apoptosis. However, although a lot of data are available on TLR4 expression in non-specific intestinal inflammation, the receptors can bind LPS, a structural component of outer membranes of Gram-negative bacteria, little is known about TLR5 expression changes in ulcerative colitis and Lesniowski-Crohn disease. The receptor may play a significant role in the initiation and sustaining the inflammatory process through TLRs stimulation and limitation of inflammatory cytokine production.

Recent studies demonstrated the presence of TLRs on tumour cells of different locations, including the colon (8). Molecular pathomechanism combining inflammatory response and carcinogenesis is not yet fully understood. Fukata et al. (9) showed TLR 4 overexpression in inflammatory processes associated with colon metaplasia. Activation of TLR4 signaling pathways caused increased prostaglandin E2 (PGE2) production as well as epidermal growth factor receptor (EGFR) activation, which is involved in enhancement of cyclooxygenase-2 (COX-2) expression shown to increase the risk of cancer development (9). The authors believe that TLR4 inhibitors might prove useful in the prophylaxis or treatment of colitis-induced carcinogenesis. It has also been demonstrated that TLR4 ligation by LPS promotes production of chemokine CCL20 that recruits immature dendritic cells (10). According to Wang et al. (10), CCL20 expressed in intestinal epithelial cells might serve as a marker to differentiate between chronic colitis and cancerous conditions. Lipopolysaccharide (LPS), a TLR4 ligand, is mentioned among basic endotoxins that can trigger the formation of distant metastases of colorectal cancer. TLR4 expression was significantly decreased in cancerous tissue of the colon in patients with distant metastases via the lymphatic or blood vessel route as compared to normal colon epithelium and non-metastatic tumours (11). TLR4 activation may help prevent the development of distant metastases of colorectal cancer; it also enhances phagocytic capacity of macrophages and increases production of reactive oxygen intermediates (ROI) and nitrogen oxide (NO) synthesis. Anti-cancer therapy with immunosuppressive drugs, eg., Rapamycin, affects the activation of nuclear transcription factor NF(κappa)B by decreasing TLR expression (12). Activation of another member of the TLR family (TLR9) by ligand CpG-DNA causes type 1 immune response. TLR9-dependent activation of lymphocytes B and dendritic cells mediates cytokine and chemokine production including IL6, IL-12, INF gamma, MIP-1, IP-10, which have the ability to destroy tumour cells (13,14). TLR9 expression was observed on colorectal cancer cells. TLR9 agonists were reported to show p53 protein-independent activity within human colorectal cancer cells, inhibit their proliferation and promote apoptosis, and improve anti-cancer effects of radio- and chemotherapy (15). Experimental studies demonstrated inhibition of tumour progression by TLR5-flagellin complex. Thus, Rhe et al. (16) propose that a TLR5-activating substance might significantly affect colorectal cancer therapy in humans. Other authors suggest a role of TLRs in colorectal carcinogenesis emphasizing that chronic inflammatory conditions are well-established risk factors in the process. Each member of the TLR family shows binding affinity to a microorganism, ie., a bacterium, virus, protozoon or fungus. Thus, an infectious factor can be involved in carcinogenesis. Some TLR family members, ie., TLR3, TLR7/8, and TLR9, are particularly involved in antiviral responses. Our study has demonstrated significant TLR3 expression variability depending on colorectal cancer stage. TLR3 expression levels in normal colon mucosa of polyposis and adenocarcinoma patients also varied between the stages of cell differentiation. Diminished TLR3 expression in adenomatous polyps may indicate increased susceptibility to viral infection than it is in the case of normal tissue. This might account for the tendency of polypoid tissue towards uncontrolled growth or even metaplasia. The normal ileal mucosa constitutively expressed TLR3 and TLR1 but no in the mucosa of active pachitis (17).
No other TLR family receptor showed so obvious variability in colon pathologies.

CONCLUSION

TLR3 may indicate the tendency of normal tissue to form polyps or colorectal cancer.

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REFERENCES


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