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ORIGINAL ARTICLE

Role of purinergic receptors in hepatobiliary carcinoma in Pakistani population: an approach towards proinflammatory role of P2X4 and P2X7 receptors

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Abstract

The primary malignancy of liver, known as hepatocellular carcinoma (HCC), comprises 9% of all hepatobiliary carcinomas. A steady rise has also been observed in adenocarcinoma (ADC) of the liver and ampullary carcinoma (AMC), ascending to 0.5% of gastrointestinal malignancies. Hepatobiliary carcinomas consist of 13% of all cancer occurrences worldwide. Purinergic receptor–based signaling holds the therapeutic potential based on its role in cell proliferation of several carcinomas. An altered ATP concentration in nanomoles may lead towards crucial changes in cancer growth patterns in liver tissue. A total of 40 tissue samples were collected (20 samples of HCC, 10 samples of ADC, and 10 samples of AMC) from patients that underwent surgery. P2X4 and P2X7 receptors exhibited significantly increased expression in HCC, ADC, and AMC samples as compared with the control tissue samples. While ADC and AMC samples showed higher expression of P2X4 and P2X7 than the control, statistically, HCC samples exhibited the most significant expression of both P2X4 and P2X7 receptors than control tissues. It may be inferred that higher expression of P2X4 and P2X7 receptors is significantly associated with the upregulated cellular stress leading to inflammation and it is plausible that both these receptors may be used in diagnostic, prognostic, and therapeutic tools for carcinoma studies in the future.

Keywords Inflammatory precursor · Purinergic signaling · Hepatocellular carcinoma · Cancer microenvironment

Introduction

With a continuous upsurge of HCC in the previous decade, it has emerged as one of the leading causes of cancer-related deaths worldwide [1]. The incidence of HCC is the highest in Asia, and especially in Pakistan, because of higher prevalence of hepatitis B and hepatitis C endemics in this region [2]. The predominant causes of HCC development include cirrhosis and chronic liver disease due to endemics of hepatitis C virus (HCV) and hepatitis B virus (HBV). Additionally, there is an increased rate of incidence for adenocarcinoma (ADC) and ampullary carcinoma (AMC). ADC is a rare malignancy in the liver caused by increased level of hormones, particularly estrogen [3]. AMC consists of biliary tract (bile duct) carcinomas that are neighboring malignancies to liver tissue. Hepatobiliary cancers, including HCC, ADC, and AMC, are the most common malignancies in Pakistan consisting of 10% of all cancers [4].

Purine-based extracellular signaling is referred to as "purinergic signaling" and it is carried through purinergic receptors [5]. Cancer microenvironment (CME) carries abundant ATP for transduction of various signals, which include induction of growth factors, danger signals, immunomodulation, and neuromodulation [6]. Purinergic receptors consist of two subdivisions: ligand-gated ion channels P2X receptors and G protein–coupled P2Y receptors [7]. P2X receptors have seven subtypes (P2X1–7) and mediate the Na^+, Ca^{2+}, and K^+ passage through the plasma membrane [8]. The role of purinergic receptors is crucial in the development of reactive oxygen species leading to chronic inflammation.

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Inflammation is a significant factor in the initiation of cancerous environment in a tissue. An aggravated expression of various inflammatory genes (e.g., NF-κB and TNF-α) has been indicated in tumor microenvironment of hepatobiliary carcinomas [9, 10]. The purinergic receptors are associated with induction of cell proliferation, apoptosis, inflammation, neuromodulation, and pain sensitivity. It is necessary to analyze purinergic receptors in order to decipher their role in hepatobiliary cancer HCC, ADC, and AMC microenvironments [11]. Two purinergic receptors, P2X4 and P2X7, are equally distributed across hepatobiliary tissues and it is suggested that they may have significant role in liver pathophysiology [12, 13].

Recently, purinergic signaling has emerged as an essential signaling mechanism to regulate inflammation [14]. Two key receptors in purinergic signaling are P2X4 and P2X7. Growing evidence reveals their influence as proinflammatory receptors in cancer progression and prognosis [15, 16]. P2X receptors' expression is involved in various pathophysiological processes during disease condition such as inflammation, visceral hypersensitivity, tumor proliferation, or apoptosis. Exposure of P2X receptors to ATP concentrations in milli-molars causes the opening of nonselective membrane pores as an ion channel [17].

In normal liver tissue, P2X receptors have been reported of depicting a low expression of P2X4 and P2X7 receptors. Previously it has been reported that induction of chronic inflammation results in overexpression of P2X receptors and consequently purinergic signaling [16]. Purinergic receptors, P2X4 and P2X7, are referred to as inflammatory precursors [18]. P2X7 receptor is mainly associated with the development of oxidative stress, subsequently leading to inflammation [16]. P2X4 receptor is involved in inflammation, fibrogenesis, apoptosis, and neuromodulation—pain sensitivity—of carcinoma [19]. Previous evidence also states that the inhibition of P2X4 and P2X7 receptors leads to a decreased inflammation in injured tissues [20, 21].

Mounting evidence of P2X receptors' expression in cancer cell indicates their significance in disease progression [7, 22]. Among different tumor types, hemopoietic lymphoproliferative disorders indicated an overexpression of P2X receptors. Lymphoproliferative disorders were the first models to be studied for P2X receptors and their association with the cancer [23]. Conversely, some carcinomas have shown reduction in the prevalence of P2X receptors as compared with the normal cells. For instance, P2X7 expression was downregulated in adenocarcinoma and cervical squamous carcinoma [24]. Overall, two hypotheses have been postulated regarding the role of P2X receptors in cancer; either the receptors play an anticancer role by inducing the P2X mediated apoptosis or the receptors function as a precancerous protein by increasing cancer cells growth and invasion [25]. P2X receptors may lead to the development of improved anticancer drugs linked to their activation or inhibition [26, 27].

The present study was designed under the objective to determine the prevalence of P2X4 and P2X7 receptors in explanted tissue samples of patients suffering from hepatocellular carcinoma, hepatic adenocarcinoma, and ampullary carcinoma using the immunohistochemistry analysis. It was hypothesized that P2X4 and P2X7 are as efficient in inflammation process of human epithelial tissues as they are in other animal models of epithelial tissues and cell lines.

Materials and methods

Tissue samples

Overall 40 specimens (20 tissue samples of HCC, 10 tissue samples of ADC, and 10 tissue samples of AMC) were acquired from 40 different patients (30 males and 10 females) and were selected for the experimental group. Preferably, control samples in our study must be from the healthy individuals but the unavailability of healthy liver tissues and denied access from the postpartum patients made us use liver biopsies from the patients that were not diagnosed with HCC. However, control biopsies were taken from patients during early stage of liver disease which includes the liver fibrosis, steatosis, and early cirrhosis. Controls represented a relative expression of antibody in comparison with selected groups of diseases, i.e., HCC, ADC, and AMC. These tissue samples were selected from the preserved tissue biopsies of patients who had undergone surgery in the year 2016 at Hepato-pancreatobiliary Liver Transplant Unit, Shaikh Zayd Hospital, Lahore (SZH). The histopathology laboratory at Liver Transplant Unit, SZH, diagnosed the carcinoma subtypes and preserved the biopsies in paraffin-embedded tissue blocks.

Etiologies of liver tissue samples comprised of HCC caused by the infection of HBV and HCV, co-infection of HBV and HCV, non-alcoholic steato-hepatitis (NASH), alcoholic hepatitis, and genetic liver disorders. ADC samples consisted of cancer of right lobe of the liver, Whipple's specimen, and biopsy of the liver. AMC samples mainly consisted of cancer of the ampulla of Vater, and pancreatic head cancer. The exclusion criteria were designed to improve the standard of the research. The patients below the age of 25 years or with procedures other than Whipple's procedure, transplant of organ, or resection of a smaller part of the organ were excluded. Additionally, the samples with metastasis were excluded to remove confounding bias.

The designed study was duly reviewed and approved by the ethics committee of Shaikh Zayd Hospital, Lahore, and Atta ur Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, in accordance with the recommendations of the World Medical Association's Declaration of Helsinki for biomedical experimentation involving human subjects.

Immunohistochemistry

The tissue blocks containing formalin-fixed, paraffin-embedded tissue samples were sectioned at a 5-μm thickness with microtome. The sectioned samples were then treated with immunohistochemistry (IHC) protocol. Xylene was first used to deparaffinize the tissue samples, which were then rehydrated with graded ethanol and immersed in phosphate-buffered saline (PBS). The samples were incubated with primary and secondary antibodies respectively for later immunostaining with chromogen. After IHC, the tissue sections were examined under a light microscope at × 100 and × 200 magnifications and more than 10 high-power fields were randomly selected.

The primary antibodies Goat pAb to P2X7 (abcam ab93354, Lot No. GR50237-11) and Goat pAb to P2X4 (abcam ab134559, Lot No. GR104930-3) were used for the experiment. The secondary antibody was "donkey primary antibody to goat IgG" (Dnk pAb Goat IgG). The dilution factor was kept 1% for both antibodies.

Scoring pattern

The following table gives an account of the basis for scoring pattern immunostained slides.

Scoring grade	Level of staining
Score 0	No staining/control samples
Score 1	Minimum staining up to 33% positive staining of cells
Score 2	From 33 to 66% positive staining of cells
Score 3	From 66 to 100% positive staining results for samples

Statistical analysis

The average expression of P2X4 and P2X7 was compared with the control group in each cancer type. Later on, P2X4 and P2X7 expressions were compared within all cancer types under study, separately. We executed one-way analysis of variance (ANOVA) to find out the significant difference among the means of different values. The statistical software GraphPad Prism 6.0 was used for the statistical analysis.

Results

Immunohistochemical analysis of P2X4 and P2X7 receptors in hepatocellular carcinoma

An elevated level of expression of the P2X4 and P2X7 receptors was observed in the samples of HCC. From 20 samples analyzed for P2X4 receptors, 12 represented a significant high expression, 5 samples represented a moderate expression, and 3 samples represented a low level of expression. The expression level of P2X7 for 20 samples included 11 significant, 5 moderate, and 4 low expression levels. These results indicate the relation of P2X4 and P2X7 receptors increased expression with inflammation. Figure 1 represents the microscopic photographs of the immunohistochemical staining of the hepatocellular carcinoma tissue samples. Figure 2 represents the bar graph for P2X4 and P2X7 receptors' expression in HCC tissues samples. The results of one-way ANOVA depict an overall occurrence of P2X4 and P2X7 receptors in HCC. The expression of both the P2X4 and P2X7 receptors was significantly higher than the control tissue samples. P2X4 was relatively higher than P2X7 in HCC tissue samples.

Immunohistochemical analysis of P2X4 and P2X7 receptors in hepatic adenocarcinoma

A significant high expression was observed for P2X4 and P2X7 receptors as compared with control tissue samples. Of the total 10 samples stained for P2X4, 5 samples represented a high expression, 4 samples represented a moderate expression, and 1 sample expressed low expression. In the case of the expression level of P2X7 receptor, 2 tissue samples exhibited a significantly high expression, 3 samples represented a moderated expression, and 5 samples expressed low expression. Figure 3 represents the microscopic photographs of the immunohistochemical staining of the hepatic adenocarcinoma tissue samples.

Figure 4 represents a bar graph of comparative expression of P2X4 and P2X7 receptors. Statistical analysis was performed and graph was plotted accordingly. The expression of P2X4 receptor is less significantly higher than that of P2X7 receptor and highly significant than the expression in control tissue while P2X7 expression falls in the mid-range, sharing a little significance with both control and P2X4 receptor expressions.

Immunohistochemical analysis of P2X4 and P2X7 receptors in ampullary carcinoma

In the case of ampullary carcinoma analysis, a moderate expression for both P2X4 and P2X7 receptors was significant. In the studied 10 samples for P2X4 expression, 3 samples showed significantly high expression, 5 samples showed moderated expression, and 2 samples represented a low expression, while the results for the P2X7 receptor expression were as follows: 5 tissue samples represented high significantly expression, 3 samples represented moderate expression, and 4 samples depicted a low expression

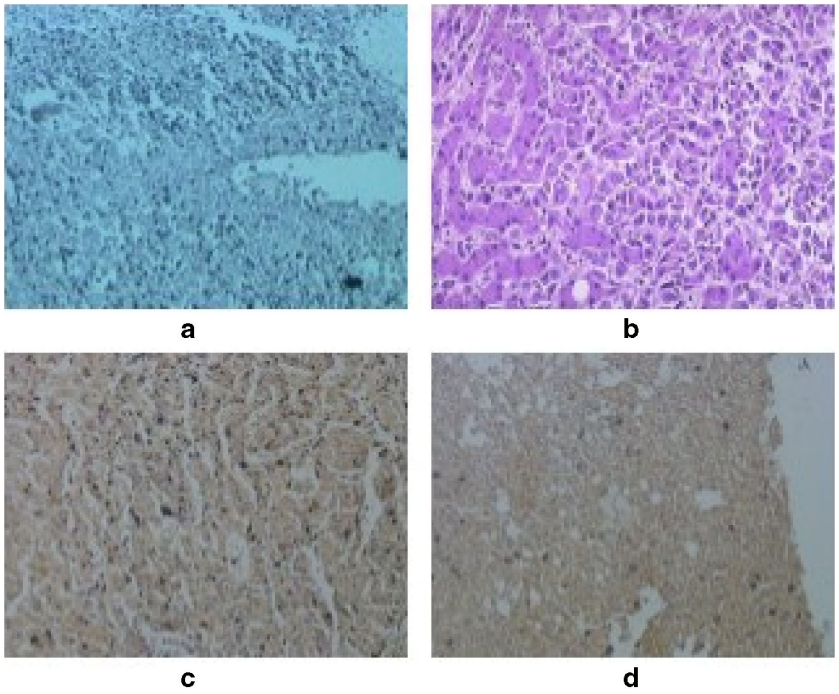

Fig. 1 Immunohistochemical staining of human hepatocellular carcinoma (HCC) tissue sections with P2X4 and P2X7. **a** Negative control of HCC tissue sample (without primary antibody). **b** H&E staining of HCC tissue sample. **c** P2X4 staining of HCC tissue sample. **d** P2X7 staining of HCC tissue sample. The figure shows representative images of the staining performed in triplicates on all samples (original magnification × 200)

of P2X7 receptor. Figure 5 represents the microscopic photographs of the staining of tissue samples of AMC.

Figure 6 represents a bar graph of statistical analysis of P2X4 and P2X7 expressions in AMC. Both receptors were expressed significantly higher than the control tissue samples; however, P2X4 and P2X7 receptors were equally expressed in the case of ampullary carcinoma with non-significant difference.

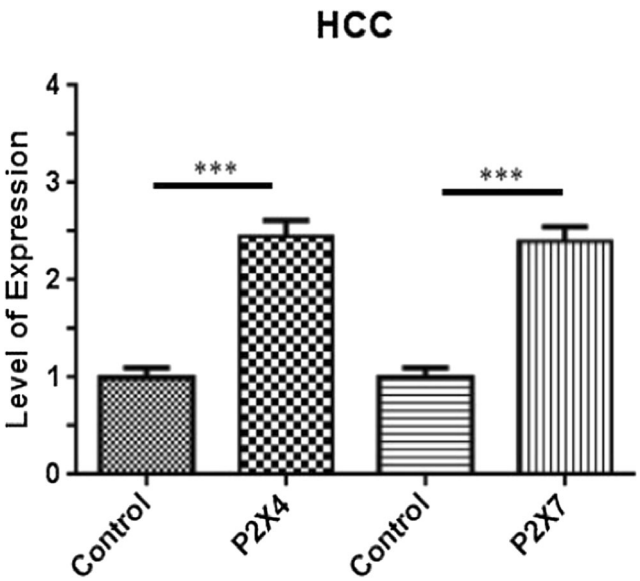

Fig. 2 P2X4 and P2X7 expressions in HCC. The expression level (*y*-axis) and receptor types (*x*-axis) have been plotted for the 20 samples of HCC tissue for P2X4 and P2X7 separately. More than 200% expression level of P2X4 and P2X7 was observed in the HCC as compared with control tissue samples. ANOVA analysis was performed in GraphPad Prism 6.0. All values are expressed in mean SEM. ***P value < 0.0001

Comparative analysis of P2X4 and P2X7 in hepatocellular carcinoma, adenocarcinoma, and ampullary carcinoma

Comparative analysis of HCC with adenocarcinoma and ampullary carcinoma represented a significant difference in the level of expression of P2X4 as well as P2X7 varied. Only 15–20% of HCC samples exhibited low expression while 55–60% of HCC samples exhibited high expression of both P2X4 and P2X7 receptors. On the other hand, adenocarcinoma and ampullary carcinoma represented varied levels of P2X4 and P2X7 receptors. Only 10% of adenocarcinoma samples expressed lower expression of P2X4 while 50% of adenocarcinoma samples expressed high level of P2X4 samples. At the same time, the expression pattern of P2X7 was quite different in adenocarcinoma. The IHC results showed a reduced expression of P2X7 with 50% of samples depicting low expression and only 20% samples depicted high expression of P2X7 receptors. Table 1 represents the percentage of P2X4 and P2X7 receptors' expression in each tissue type.

Again, in the case of ampullary carcinoma, the expression of P2X4 and P2X7 comes in close range to each other. In ampullary carcinoma, a moderate expression trend was observed. A low expression of P2X4 was observed in 20% of samples of AMC and low expression of P2X7 was observed in 40% of samples of AMC. A total of 50% samples expressed a moderate expression of P2X4, while 30% of samples expressed a moderate expression of P2X7 receptors. Interestingly, there was equal percentage of high expression of P2X4 and P2X7 receptors, up to 30% of the samples with high expression.

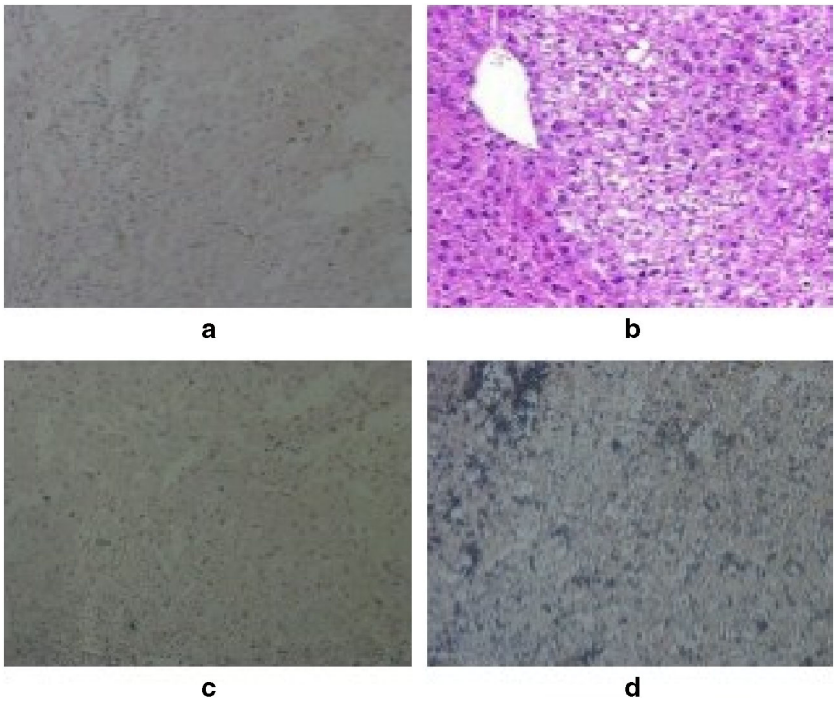

Fig. 3 P2X7 and P2X4 expressions upon adenocarcinoma samples. Immunohistochemical staining of human hepatocellular adenocarcinoma (ADC) tissue sections with P2X4 and P2X7. **a** Negative control of ADC tissue sample (without primary antibody). **b** H&E staining of ADC tissue sample. **c** P2X4 staining of ADC tissue sample. **d** P2X7 staining of ADCC tissue sample. The figure shows representative images of the staining performed in triplicates on all samples (original magnification × 200)

Discussion

Recently, cancer microenvironment has acquired increased scientific attention. It harbors key molecules to modulate signaling patterns influencing cellular fate. ATP is one of the most critical molecules affecting cell growth patterns by stimulating purinergic receptors mainly P2X ion channels. This preliminary study was designed to analyze the qualitative expression of P2X4 and P2X7 receptors in hepatobiliary carcinoma, i.e., HCC, ADC, and AMC.

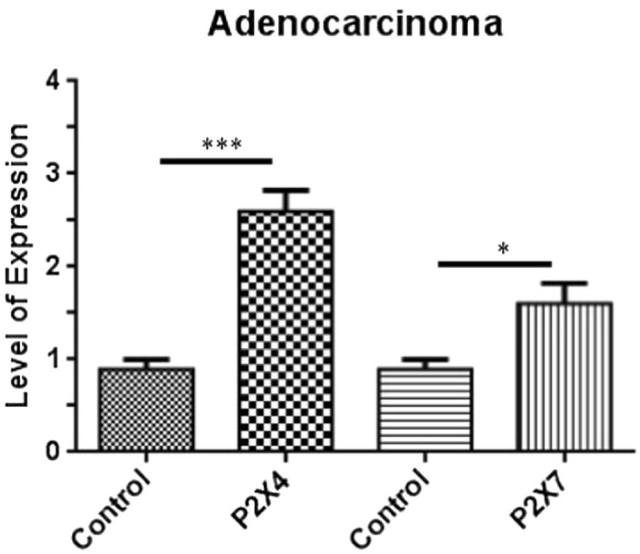

Fig. 4 P2X4 and P2X7 expressions in adenocarcinoma. The expression level (*y*-axis) and receptor types (*x*-axis) have been plotted for the 10 samples of ADC for P2X4 and P2X7 receptors separately. As compared with the control samples, P2X4 receptor expression was significantly high with more than 200% increase while the expression of P2X7 receptor was about 100% higher than that of the control samples. ANOVA analysis was performed in GraphPad Prism 6.0. All values are expressed in mean SEM. ***P value < 0.0001. *P value < 0.05

A low expression of P2X4 and P2X7 was assessed in control tissue samples, which was comparatively equal in all three cancer types. This histopathological examination aligns with previous evidence that establishes the comparatively equal occurrence of P2X4 and P2X7 receptors in hepatocytes. While assessing the carcinoma tissue samples, the average expression values of P2X4 and P2X7 increased vigorously, this aligns with the recent evidence to strengthen the theory of P2X4 and P2X7 receptors' association with incessant inflammation. A relatively gradual increase in their expression with the development of disease has been found substantial in multiple cancer types [14, 25, 28, 29]. The P2X4 and P2X7 receptors' expression for a specific cancer type in the studied carcinomas was relatively equal with a nominal difference which is affirmed by a recent evidence of P2X4 and P2X7 receptors' co-expression in the form of hetero-trimers on the cell surface [30–32]. In spite of a minor difference of expression between P2X4 and P2X7 within specific tissue type, the absolute level of fluorescence may be dependent on the properties of the antibodies.

On the contrary to specific cancer-type analysis, the comparative analysis of HCC, ADC, and AMC reveals that HCC represents the highest expression level of P2X4 and P2X7 receptors among them. Our assessment of P2X4 expression analysis exhibits a significant rise above the moderate range for all the studied cancer tissue samples, in which HCC expressed the highest amount. Likewise, P2X7 receptor expression in HCC was found the highest among all three cancer

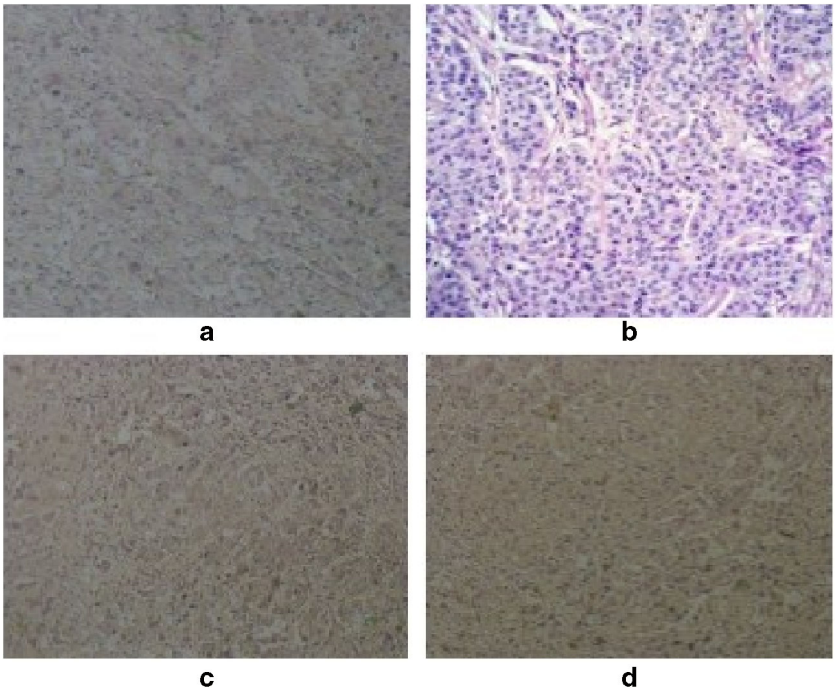

Fig. 5 Ampullary carcinoma expressing P2X7 and P2X4 receptors. Immunohistochemical staining of human ampullary carcinoma (AMC) tissue sections with P2X4 and P2X7. **a** Negative control of AMC tissue sample (without primary antibody). **b** H&E staining of AMC tissue sample. **c** P2X4 staining of AMC tissue sample. **d** P2X7 staining of AMC tissue sample. The figure shows representative images of the staining performed in triplicates on all samples (original magnification × 200)

types. The mounting evidence of extracellular ATP's role in CME makes it one of the main driving factors for modulation of P2X expression; we suggest that ATP accumulation in the CME of ADC and AMC is lower than that of HCC that may be a responsible factor in reducing the expression of P2X4 and P2X7 receptors in ADC and AMC tissue samples.

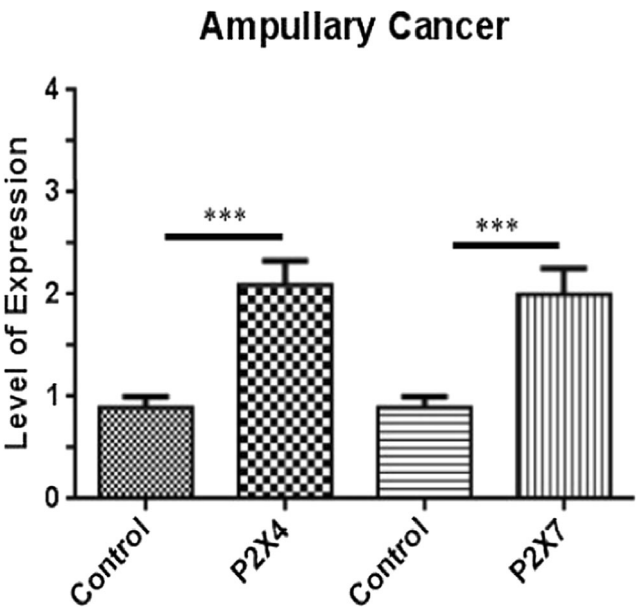

Fig. 6 P2X4 and P2X7 expressions in ampullary carcinoma. The expression level (*y*-axis) and receptor types (*x*-axis) have been plotted for the 10 samples of ADC for P2X4 and P2X7 receptors separately. There was a significant increase in the expression of P2X4 and P2X7 receptors in AMC. ANOVA analysis was performed in GraphPad Prism 6.0. All values are expressed in mean SEM. ***P value < 0.0001

Although ADC and AMC exhibited a significantly higher expression of P2X4 and P2X7 than in their respective control tissues, ADC and AMC exhibited a lower expression in comparison with HCC. This significant variance presented by HCC towards ADC and AMC may be due to the fact that prolonged inflammation period increases ATP concentration in CME and mediate increased expression of purinergic receptors [33, 34]. It may also suggest that liver tissue may harbor a higher quantity of infiltrated immune cells. These immune cells further increase the ATP levels in CME, thus leading towards higher activation of P2X receptors in HCC.

P2X4 expression was the highest in HCC tissue. The difference of P2X4 expression in ADC and AMC was nominal. In contrary, HCC exhibited significantly high P2X7 ratios when compared with ADC and AMC tissue samples. Recent evidence suggests that higher P2X4 expression in HCC is also influenced by viral invasion [35]. HCC tissue samples also exhibited the highest expression of P2X7 receptor among all three carcinoma samples. From liver scarring to the development of HCC, it takes an average of 5 to 10 years to develop, while ADC and AMC are subjected to a sudden genetic change or a shorter inflammatory period with an exception of metastatic development of HCC [36, 37]. This may dictate that the duration of carcinoma development is also an important factor in the varied expression levels of P2X receptors found in our study.

Similar to P2X4, the expression difference of P2X7 in ADC and AMC is nominal. Statistically, P2X7 in HCC had extremely significant difference from ADC and a slight

Table 1 Total number of samples falling in specific grading pattern

Samples	Significance of P2X4 expression			Significance of P2X7 expression		
	Low (%)	Moderate (%)	High (%)	Low (%)	Moderate (%)	High (%)
HCC	15	25	60	20	25	55
Adenocarcinoma	10	40	50	50	30	20
Ampullary carcinoma	20	50	30	40	30	30

significant difference from AMC samples. It is suggested that with a longer duration of inflammation in carcinoma development, the expression of P2X4 and P2X7 increases as is the case of HCC development.

The expression of P2X7 in the studied samples highlights a complex pattern with subject to different tissues as compared with P2X4 receptor, indicating more diverse role of P2X7 receptor. Our study aligns with the previous findings of disordered purinergic signaling in colorectal cancer that highlighted that increased tumor volume led to a higher expression of CD39 [38]. Subsequently, we suggest tumor volume as one of the potential factors linked with increased P2X receptor expression in HCC as compared with ADC and AMC samples.

Previously, gender bias has been reported in gastrointestinal carcinomas due to the interplay of hormones with carcinoma progression [39]. The relation of P2X4 and P2X7 receptors' expression with the age and gender of patients was statistically insignificant and it suggests an independent expression trend.

Continuous evidence suggests that higher expressions of purinergic receptors assist in proliferation. Higher stimulus to P2X receptors has shown downstream signaling associated with reduced apoptosis and increased expression of proliferation biomarkers in various carcinoma tissues [40]. Our study affirms the hypothesis of gradual increase in the expression of both P2X4 and P2X7 proteins with a potential linkage in inflammation. Since previous studies of P2X4 and P2X7 receptors' expression were only executed on animal models and cell lines of HCC and there are no previous findings in ADC and AMC on P2X4 and P2X7 expression, thus, the statistically significant expression highlights the influence of purinergic receptors for the first time in human carcinoma tissue samples. In conclusion, both P2X4 and P2X7 may be attributed as decisive inflammatory markers to define cellular growth pattern.

The results represented statistical relevance between the expression level of P2X4 and P2X7 receptors. It may be inferred from the results that P2X4 and P2X7 receptors are strongly associated with the downstream process of inflammation by activation of inflammasome, oxidative stress, and immune modulation for continuous cancer progression. Furthermore, we can draw the conclusion that both receptors have a direct correlation with inflammation and tumor mass, and the level of expression proportionally elevates with disease progression. These biomarkers have future potential to be used as diagnostic, prognostic, and therapeutic tools in cancer research.

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Compliance with ethical standards

The designed study was duly reviewed and approved by the ethics committee of Shaikh Zayd Hospital, Lahore, and Atta ur Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, in accordance with the recommendations of the World Medical Association's Declaration of Helsinki for biomedical experimentation involving human subjects.

Conflict of interest Arun Asif declares that he has no conflict of interest.
Madiha Khalid declares that she has no conflict of interest.
Sobia Manzoor declares that she has no conflict of interest.
Hassam Ahmad declares that he has no conflict of interest.
Aman-ur-Rehman declares that he has no conflict of interest.

Research involving human participants and/or animals This article involved liver biopsy samples from human participants, and does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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