Impact of AKT1 polymorphism on DNA damage, BTG2 expression, and risk of colorectal cancer development

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Background. AKT, also called protein kinase B, is a serine-threonine kinase that functions as a mediator of PI3K-Akt-mTOR signaling pathway and plays an important role in an array of cellular processes. Many single nucleotide polymorphisms (SNP) in AKT gene have been observed to be associated with various types of cancers. In the current research the association of a functional SNP rs1130233 in AKT, depicting G to A transition, was studied with AKT activation, DNA damage, an early response B-cell translocation gene 2 (Btg2) expression and risk of colorectal cancer (CRC) development.

Patients and methods. A total 197 population-based controls and 200 CRC patients were genotyped for SNP rs1130233. AKT expression, activation and BTG2 expression were determined in GG, AG and AA genotype carriers. DNA damage was determined through comet assay.

Results. The heterozygous AG genotype (55.67%) was more prevalent in the local population compared to homozygous wild type GG (37.78%) and homozygous AA genotypes (6.55%). Moreover, AG and AA alleles were observed to be significant contributors (P = 0.01, OR = 1.80, CI = 1.18 to 2.74, and P = 0.001, OR = 5.00, CI = 1.90 to 13.18, respectively) in increasing the risk of CRC. The immunoblot analysis revealed that G to A transition decreased the expression and activation of AKT. Moreover, AG and AA genotypes of AKT1 rs1130233 showed a significant increase in DNA damage and Btg2 expression.

Conclusions. The data concludes that G to A substitution is a risk factor for CRC development involving a decrease in AKT expression and activation and increase in DNA damage.

Key words: AKT1; BTG2; colorectal cancer; DNA damage; rs1130233

Introduction

Colorectal cancer is a multifactorial disease, and its life time risk in the general population increases ~5% with age.1 This may be caused by carcinogenic compounds ingestion through foods and importantly individual differences in the metabolism of carcinogens as caused by both genetic and environmental risk factors which play essential roles in the development of colorectal cancer. Many genes sequence variations lead to the pathogenesis of inherited and sporadic forms of colorectal cancer.2,3 AKT, also known as Protein Kinase B (PKB), is a serine/threonine protein kinase which was originally discovered as an oncogene transduced by the acute transforming retrovirus (PKB-8/AKT8), isolated from mouse leukemia.4,5 AKT is the downstream target of PI3K signaling that triggers a number of biological processes including cell survival, cell growth, glucose metabolism, angiogenesis, cell cycle entry cell motility, and also stimulate malignant transformation of cells and tumor progression. PI3K/AKT/mTOR pathway is one of the central nodes in many physiological abnormali-
ties including cancer. Mammalian AKT gene has three isoforms; AKT1, AKT2, and AKT3. All these isoforms show broad tissue distribution and a broad range of functions. AKT1 also known as AKT kinase, is ubiquitously expressed isoform.

Constitutive activation of AKT is mainly attributed to the aberrant activation of upstream signaling such as mutation or hyperactivation of receptor tyrosine kinases (Src, Ras and PTEN proteins) and increased synthesis of growth factors, as has been observed in several types of cancers. Genetic variations in AKT gene (e.g. rs1130233, rs2498801, rs2494752) is linked with various types of cancers including liver, lungs and bladder cancers. Genetic polymorphic forms of AKT differ widely in their role as oncogene and exert their actions by regulating a diverse array of genes including NF-kB, Btg2 etc.

Human B-cell translocation gene 2 (BTG2), an ortholog of mouse TIS21, is a tumor suppressor gene that belongs to an antiproliferative gene family. BTG2 is implicated in a variety of physiological processes including cell differentiation, development, cell cycle arrest at G1/S and G2/M phases, increased synthesis of growth factors, as has been observed in several types of cancers.

Patients and methods

Patients

This case-control study involved a total of 397 individuals including both colorectal cancer patients (CRC; n = 200) and population-based controls (n = 197). CRC patients (n = 200) from both sex, having age ≤ 60 and with documentary evidence of pathologically confirmed adenocarcinoma of colorectal cancer were included in this study. Subjects with mixed ethnic background, comorbidity, and patients who developed CRC at the age of above 60 years at the diagnosis were excluded. Determination of tumor stages and types were done by experienced pathologist at Institute of radiation and nuclear medicine (IRNUM), Peshawar. All patients and their guardians were informed about the nature of the study and important information of patients such as age, sex, ethnicity, medical records, pathology reports, drug history, family history, tumor size, tumor location and lymph node status etc. were obtained on a pre-designed proforma. Colorectal cancer risk factors such as taking red meat, vegetables, fibers, fruits and cooking choices and smoking history were also obtained. For control blood samples were collected from healthy individuals (n = 197) who had no sign of present or previous malignancy and no indication of CRC or nor any family history of cancer and had no blood relation with patients. Selection of control group of healthy donors was done on the basis of sex, age, smoking history and habits, residential, occupational and food intake. Informed consent of all the enrolled subjects was obtained on a questionnaire. The ethical approval was obtained from the institutional ethical board at Department of Biotechnology, University of Peshawar, Pakistan.

Blood samples were collected both from colorectal cancer patients and controls at IRNUM Peshawar in 5 mL EDTA tubes and were stored at -20°C till further analysis.

DNA extraction and genotyping

DNA was extracted using DNA extraction kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, USA) and was quantified using UV-visible spectrophotometer (752 PC, China). Akt single nucleotide polymorphism was determined using polymerase chain reaction (PCR, Multigene Optimax, Labnet International, USA). PCR was performed in a 20 μL reaction mixture using allele specific primers. The sequences of primers and amplification condition are given in Table 1. The AA (379 bp), AG (245 and 379 bp) and GG (245 bp) genotypes were visualized with ethidium bromide and identified on agarose gel (2%) using UV transilluminator (Wooltec, USA).
Isolation of lymphocytes

Lymphocytes were isolated from fresh blood as described previously. Briefly, blood containing EDTA was mixed with phosphate buffer saline (PBS; Ca²⁺ and Mg²⁺ free) and layered over 2 mL ficoll / lymphocytes separation medium (LSM™1077; Catalog Number: HiSep LSMTM 1077-LS001) in a 15 mL falcon tube. The mixture was centrifuged (2000 RPM for 30 min) that led to the formation of four distinct layers; the upper plasma layer, the second buffy coat layer containing lymphocyte and monocyte, the third ficoll layer (LSM) and the bottom layer of RBCs and cell debris. The buffy coat was isolated, mixed with 1 mL PBS and centrifuged at 1500 RPM for 10 min. The pellet containing isolated lymphocytes washed with PBS, gently suspended in 1 mL PBS and used in subsequent experiments.

Comet assay for DNA damage

DNA damage in lymphocytes was assessed using comet assay, (also called single cell gel electrophoresis assay), as described previously. Briefly, cells were fixed in ethanol for 20 min, then hydrated in distilled water for 30 min followed by staining. The slides were washed with cold distilled water and mounted with the cover glass. For scoring of DNA comets, 100 stained nuclei were selected randomly from each group under the fluorescent microscope at 200x magnification and images were recorded. Total comet score was calculated as described previously.

Immunoblot analysis

The isolated lymphocytes were lysed in a buffer containing Tris (50 mM, pH 7.4, NaCl (150 mM), EDTA (1.0 mM), phenylmethylsulphonyl fluoride (1.0 mM), aprotinin (1.0 μg/ml), leupeptin (1.0 μg/ml), NaF (1.0 mM, 1.0 mM) sodium orthovanadate, sodium deoxycholate (0.25 %) and Nonidet P-40 (1.0%). The extracted proteins were quantified and electrophoresed on SDS-PAGE, transferred onto a nitrocellulose membrane using immunoblotting kit. Membrane was incubated with anti-Akt, anti-pAkt and anti-tubulin and proteins were detected using immunoblotting detection kit Ab Signal™ (AbClon, Seoul, Republic of Korea). Antibodies for AKT and pAKT were purchased from Cell Signaling Technology while α-tubulin from Santa Cruz Biotechnology. α-Tubulin was used as a loading control.

RNA extraction and polymerase chain reaction (PCR)

Total RNA was extracted from purified PBMCs using Trizol reagent. RNA was reverse transcribed to cDNA using reverse transcription kit (Invitrogen). BTG2 expression was determined using conventional PCR followed by agarose gel electrophoresis. GAPDH was used as an endogenous control. The primer sequences and amplification conditions for BTG2 and GAPDH are given in Table 1.

Statistical analysis

Data was analysed using Minitab® 17 and was presented as Mean ± SD. Odds ratio (OR), 95% confidence interval (CI) were used to find out the association between AKT1 single nucleotide polymorphism and CRC risk. P ≤ 0.05 was considered as statically significant.

Results

Association of Akt1 rs1130233 with risk of colorectal cancer development

Frequency of selected demographic and risk factors in CRC cases and controls

A total of 200 CRC patients and 197 age, and sex matched CRC free healthy subjects were enrolled.

| Genes | Direction | Primer sequence | Amplification condition |
|-------|-----------|-----------------|-------------------------|
| BTG2  | Forward   | 5’-CCITGGGCAGAGAGTGAAG-3’ | 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 45 s, 72°C for 45 s and 72°C for 10 min |
|       | Reverse   | 5’-CCTTCATCACTAACCCCAT-3’ | |
| GAPDH | Forward   | 5’-CCATGGAGAGGCCTGGG-3’ | 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 45 s and 72°C for 10 min |
|       | Reverse   | 5’-CAAGATGTCGCTAAGACC-3’ | |
| AKT1  | Forward   | F1-5’-ATAGGGAGTCATGGAGGTTT-3’ | |
|       | Reverse   | R1-5’-CTTACAAAATCCTGGCAGTAAG-3’ | |
|       | Forward   | F2-5’-AAAAATTTGATGAGGAGGAAG-3’ | |
|       | Reverse   | R2-5’-TAATCCCTGCCCTGCTAC-3’ | |
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The data about the demographic information is given in Table 2. Among 200 clinically diagnosed CRC cases, there were 81 (59.50%) female and 119 (40.50%) male patients which shows that in female population of Khyber Pakhtunkhwa CRC frequency is relatively less than males as indicated by the higher incidence of CRC among males. There were 85 (42.50%) women and 112 (57.50%) men among the control group. The age and sex related differences were non-significant between the CRC and control groups (P > 0.05). The smoking status indicated that most of the subjects, including both patients and control, were non-smokers and non-significantly different in case and control cohorts (P > 0.05). Food intake especially vegetables consumption plays an important role in maintaining proper health, however, with regard to vegetable consumption the difference between control and patients was non-significant (P = 0.249). The family history data indicate that the prevalence of CRC was not linked with family history of any type of cancer. All CRC patients were divided into four groups based on Tumor Node Metastasis (TNM) staging criteria; where patients with stage I: 1 (0.50%); stag II: 33 (16.50%); stage III: 112 (56.00%), and stage IV: 54 (27.00%).

**Frequencies of Akt1 rs1130233 polymorphism and alleles distribution in colorectal cancer patients and control**

Overall 397 subjects including 200 colorectal cancer patients (cases) and 197 healthy individuals (control) were enrolled in this study and genotyping of AKTI rs1130233 was performed to evaluate the status of rs1130233 polymorphism in case and control groups. Representative images of wild type GG, heterozygous mutant AG and homozygous mutant AA alleles for both control and CRC patients are given in Figure 1. The data has been presented in Table 3. Among 200 colorectal cancer patients, 60 (30.0%) had wild type GG genotype, 120 (60.0%) had heterozygous mutant AG genotype while remaining 20 (10.0%) had homozygous mutant AA genotype. In control population, 90 subjects (45.69%) had GG genotype, 101 (51.27%) had AG and 6 (3.04%) had AA genotypes. The presence of AG and AA alleles were associated with
colorectal cancer risk (OR = 1.80, CI = 1.18–2.74, P = 0.01 for AG and OR = 5.00, CI = 1.90–13.18, P = 0.001 for AA). The association between AKT1 rs1130233 polymorphism and colorectal cancer was also assessed using dominant and recessive models. For dominant model (GG vs AG+AA), homozygous wild type (GG) was present in 60 patients (30.0%) and 90 controls (45.69%) while heterozygous and homozygous mutant alleles (AG+AA) were collectively present in 140 patients (70.00%) and 107 controls (54.31%). An increased risk for colorectal cancer (OR = 1.96, CI = 1.30–2.96; P = 0.001) was observed for dominant model. For recessive model, (AA vs GG+AG), homozygous polymorphism was observed in 20 patients (10.00%) and 6 controls (3.04%) while homozygous wild type and heterozygous polymorphism (GG+AG) were collectively present in 180 patients (90.00%) and 191 controls (96.96%). An increased risk for colorectal cancer was also observed for recessive model (OR = 3.85, CI = 1.38–10.73; P = 0.01) models.

The association between AKT1 rs1130233 polymorphism and rectum cancer was also evaluated (Table 5). Among 98 rectum cancer patients, GG, AG and AA genotypes frequencies were 30.61, 60.20, and 9.19% respectively. Both AG (OR = 1.75, CI = 1.04–2.96; P = 0.04) and AA (OR = 4.50, CI = 1.48–13.69; P = 0.008) were associated with higher risk for development of rectum cancer. An increased risk for rectum cancer (OR = 1.91, CI = 1.14–3.18; P = 0.01) was observed for dominant and recessive models (OR = 3.22, CI = 1.11–9.32; P = 0.03).

**Association of AKT1 rs1130233 polymorphism with tumor location**

On the basis of tumor location, the colorectal cancer patients were separated as colon and rectum cancer patients and the association of AKT1 polymorphism was assessed. Among overall 200 colorectal cancer patients, 102 patients (51%) had colon cancer while 98 patients (49%) were rectum cancer patients. Among colon cancer patients, GG, AG and AA genotypes frequency were 29.41, 59.80 and 10.79% respectively. The AG and AA genotypes were associated with higher risk for development of colon cancer (OR = 1.81, CI = 1.08–3.05; P = 0.02 for AG and OR = 5.50, CI = 1.87–16.15; P = 0.001 for AA). An increased risk for colon cancer was observed for dominant (OR = 2.02, CI = 1.21–3.36; P = 0.006) and recessive (OR = 3.85, CI = 1.38–10.73; P = 0.01) models.

**Frequencies of AKT1 rs1130233 polymorphism and alleles in colon cancer cases and control**

The colorectal cancer patients were sub grouped into colon and rectum cancer patients and their association AKT1 rs1130233 polymorphism was determined (Table 4). Among overall 200 colorectal cancer patients, 102 patients (51%) were colon cancer patients while 98 (49%) were rectum cancer patients. Among colon cancer patients, GG, AG and AA genotypes frequencies were 29.41, 59.80 and 10.79% respectively. The AG and AA genotypes were associated with higher risk for development of colon cancer (OR = 1.81, CI = 1.08–3.05; P = 0.02 for AG and OR = 5.50, CI = 1.87–16.15; P = 0.001 for AA). An increased risk for colon cancer was observed for dominant (OR = 2.02, CI = 1.21–3.36; P = 0.006) and recessive (OR = 3.85, CI = 1.38–10.73; P = 0.01) models.

**TABLE 3. Gene and allele frequencies of AKT1 rs1130233 polymorphism and its association with colorectal cancer**

| Type of polymorphism | Genotype | Cases n = 200 (%) | Control n = 197 (%) | P value | OR (95% CI) |
|----------------------|----------|------------------|---------------------|---------|-------------|
| Genotype Frequency   | GG       | 60 (30.00)       | 90 (45.69)          | Reference |
|                      | AG       | 120 (60.00)      | 101 (51.27)         | 0.01     | 1.80 (1.18–2.74) |
|                      | AA       | 20 (10.00)       | 6 (3.04)            | 0.001    | 5.00 (1.90–13.18) |
| Dominant Model       | GG       | 60 (30.00)       | 90 (45.69)          | 0.001    | 1.96 (1.30–2.96) |
|                      | AG+AA    | 140 (70.00)      | 107 (54.31)         |          |              |
| Recessive Model      | GG+AG    | 180 (90.00)      | 191 (96.96)         | 0.01     | 0.28 (0.11–0.72) |
|                      | AA       | 20 (10.00)       | 6 (3.04)            |          |              |
| Allele Frequency     | G        | 0.6000           | 0.7132              |          |              |
|                      | A        | 0.4000           | 0.2868              |          |              |

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patients (36.74%) had GG genotype, 55 patients (56.12%) had AG genotype while remaining 7 patients (7.14%) had AA genotype. The AG and AA polymorphism had equal effect on the prevalence of both colon and rectum cancer as both of them showed non-significant difference (P > 0.05) for AG and AA transition.

**Effect of rs1130233 on AKT protein expression and phosphorylation**

To find out whether rs1130233 G to A transition can have effect on AKT expression, lymphocytes were isolated from various subjects of different genotypes (GG = 21, AG = 25 and AA = 06) and their AKT and pAKT proteins levels were determined using immunoblotting. The data indicated that G to A transition decreased AKT expression in both healthy in various individuals independent of their age, sex and health status (Figure 2A). The densitometry analysis revealed GG genotype carriers had significantly (P < 0.05) higher level of AKT followed by heterozygous AG carriers while the AKT expression was lowest in AA genotypes (Figure 2B). pAKT represents the active kinase, therefore, the phosphorylation status of AKT was also determined in GG, AG and AA carriers. pAKT level also showed a decreased intensity in GG>AG>AA order (Figure 2C). The data shows that substitution of G by A have a significant impact on AKT expression and activation and hence could have an effect on colorectal cancer development in different ways.

**Association of AKT1 rs1130233 with DNA damage**

To find out the association of rs1130233 with genome integrity, DNA damage was assessed by comet assay. Because cancer patients have multiple genes mutation leading to DNA damages, therefore comet assay was performed only in control individuals carrying GG (n = 13), AG (n = 15) or AA (n = 4) alleles. Because age and life style can have an impact on DNA damage, therefore comet assay was performed in individuals of similar age groups, non-smoking subjects and subjects with similar dietary habits. Moreover, the frequency of AA genotype carriers in control individuals was very less, therefore, the combined total comet score was calculated for AG and AA genotype carriers (AG+AA) (Figure 3). The total comet score for individuals carrying AA genotype was 190 ± 30.5, AG genotype (110 ± 20.54) and GG 63 ± 15.70. The total comet score of AA genotypes was significantly (P < 0.05) greater than AG and GG genotypes. Also, AG carriers had significantly higher (P < 0.05) comet score than GG genotype indicating a greater DNA damage. The data thus indicates that GG allele of AKT1 contributes to genome stability.
Association of AKT1 rs1130233 with BTG2 expression

Previously we have reported that AKT downregulates BTG2 expression in various types of cells. Moreover, BTG2 has been shown to be involved in DNA damage repair, we therefore determined the expression of BTG2 in GG (n = 20), AG (n = 24) and AA (n = 6) carriers of AKT1 rs1130233 single nucleotide polymorphism. BTG2 expression was determined in lymphocytes of control individuals of similar age groups and life style. The BTG2 expression profile and its densitometric analysis is given in Figure 4. The data shows that an inverse association between AKT activation and BTG2 expression in genotype dependent manner. BTG2 expression was significantly (P < 0.05) higher in AA and AG carriers compared to GG individuals indicating that various genotypes of AKT1 differentially regulate Btg2 gene expression and hence will impart distinct effect of various cellular processes.

Discussion

CRC is a multifactorial disease. Exposure to environmental toxins, life style and internal factors including genetic variations are important factors responsible for CRC development. It has been demonstrated that lifestyle factors, including diet has a significant association with risk of CRC. Dietary pattern contributes to risk of CRC and mortality among CRC survivors. Higher intake of red and processed meat is associated with increased risk of CRC, while higher intake of vegetables, whole grains, dairy products, and fish show inverse associations with CRC risk. In the current research project, vegetable consumption was however, not significantly associated with CRC risk, as nearly all patients were from low economic background.
who most of the time rely on vegetable sources for their daily diet. Moreover, the smoking behavior in the current population is in general less and hence smoking was also a non-significant contributor to CRC risk in the current model, as most of the patients were non-smokers.

The genetic factor involving genes sequence variations have been linked with an increased risk for various types of cancers. AKT has a key role in controlling various cellular functions like cell growth, proliferation, DNA damage repair and cell survival etc. Various research based evidences suggest that AKT is activated in various types of cancers. Furthermore, genetic variations in AKT are reported to affect AKT functioning and hence can have a crucial role in tumorigenesis. Genetic variations have been linked with an increased risk of CRC patients being associated with the A allele presence. Furthermore, AKTI rs1130233 G/A+A/A genotypes have been observed to favor apoptosis, resulting in the higher risk of muscle atrophy and cachexia and weight loss in human cachexia cancer. The underlying mechanism involves the increased production of inflammatory cytokines in patients who suffer from tumor induced inflammation.

Various genetic variations of AKTs, such as single nucleotide polymorphisms (SNPs), have also been well recognized to modulate gene function. The G to A substitution significantly decreased AKTI expression and phosphorylation. The AKTI rs1130233 polymorphism is located in exon 8 and the G→A variation is located at the boundary of exon 8 and intron 7. Because of this unique localization the G to A transition interferes the posttranscriptional modification of AKTI gene leading to decrease in its expression that in turn causes low AKTI protein synthesis and activation.

AKTI rs1130233 (AG and AA genotypes) was observed to be linked with an increase in DNA damage. There are however, conflicting reports about the role of AKTI in DNA damage. The downregulation of the PI3K-AKT/ mTORC1/ p70S6K pathway has been observed to have profound effects on genome stability via suppression of MRE11 expression leading to escalation of Ras-induced DNA damage. Gol et al. has shown that both AKTI and AKT2 isoforms are involved in radiation induced-DNA double strand break repair through homologous recombination in colon cancer cells. Because AG and AA genotypes are characterized by a decrease in AKTI activation (phosphorylation), which in turn leads to an increase in genome instability and hence provides a possible link between AG and AA genotypes and associated DNA damage.

AKTI is shown to exert its effects through various mediators, such as protein kinases and phosphatases, survival factors, regulators of protein synthesis etc. Previously we have reported that AKTI increases cells survival and proliferation of cancer cells through downregulation of BTG2 expression. The current study shows a strong correlation between BTG2 upregulation and a decreased in AKTI expression and activation as depicted in AG and AA carriers. BTG2 gene has also been shown to be upregulated in response to DNA damage and hence acts as a marker of DNA damage and repair pathway.
In the current research a decreased in AKT expression and activation is linked with an increase in DNA damage indicating an important mechanism for BTG2 upregulation. However, more work is required to underpin this signaling mechanism. The AA genotype of AKT rs1130233 is present at a low frequency, therefore a large set of population is required to further confirm the impact of AA allele in CRC. AKT is widely employed in a number of different types of cancers and it is important to determine the association of AKT rs1130233 polymorphism with other types of cancers also. Moreover, how G to A transition in AKT rs1130233 effects posttranscriptional modification of AKT needs to be addressed.

Conclusions
The present study concludes the possibly important role of Akt1 in the development of colorectal cancer. The study determined that AKT1 rs1130233 polymorphism is a risk factor for the development of colon and rectum cancers and is significantly associated with DNA damage.

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