APC, BRAF and KRAS mutations, and MLH1, MGMT and CDKN2A expression analysis in Nepalese colorectal cancer patients.

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ABSTRACT

Colorectal cancer (CRC) is a common malignancy which develops due to old age and lifestyle factors, low percent of patients afflicted by a genetic disorders. Half of all colorectal cancer patients are diagnosed after metastasis. The high rate of the late detection, emphasizes on the requirement of convenient and inexpensive diagnostic methods for comprehensive screening programs. The aim of this study was to discover proto-oncogenes mutation and assessment of tumor suppressor genes expression. Formalin fixed paraffin embedded (FFPE) histologically verified colorectal cancer samples were used. APC, KRAS and BRAF mutations were investigated using polymerase chain reaction (PCR) fragments and direct sequencing. Gene expression assessment of MLH1, MGMT and CDKN2A were achieved via quantitative polymerase chain reaction (qPCR). In the present study we could detect a novel transversion heterozygous mutation in APC gene codon 1365 in three patients. BRAF codon 600 mutation were detected in one patient. KRAS codon 12 mutation was discovered in one sample and also a novel transition mutation in codon 15 was detected in 6 patients. In 80% of cases, MLH1 and MGMT expression were undetectable, in remaining 20%, MLH1 expression were reduced, but MGMT showed both reduced and increased expression compared to control. In 100% of patients CDKN2A expression was undetectable. The rate of mutations in predetermined hotspot codons and amount of uncommon mutations into APC, BRAF and KRAS in Nepalese patients indicates the requirement of further investigation in CRC patients from that part of the world. Also, the expression rate of MLH1, MGMT, CDKN2A and deficiency of an information source emphasizes the necessity of whole genome CRC expression profiling data to comparison and conclusion.

Keywords: Colorectal cancer (CRC), Formalin fixed paraffin embedded (FFPE), APC, KRAS, BRAF, polymerase chain reaction (PCR), MLH1, MGMT, CDKN2A, quantitative polymerase chain reaction (qPCR), hotspot, expression profiling.
Popular scientific summary

Colorectal cancer (CRC) is a common malignancy which develops due to old age and lifestyle factors, half of all colorectal cancer patients are diagnosed after metastasis. Available screening tests for colorectal cancer consist of visual (structural) exams and stool-based tests. Stool-based tests are finding cancer in early stage and before metastasis, often allows for more treatment options. Stool-based diagnostic tests include a multi-targeted stool DNA test (MT-sDNA). The DNA test consists of quantitative molecular tests for 10 DNA biomarkers, including 7 KRAS point mutations. This project tried to discover uncommon mutations in tumor suppressor and proto-oncogenes especially KRAS. Interestingly, two novel mutations detected into APC codon 1365 and KRAS codon 15 which similar mutations have been reported just in the South Asia region in Chinese and Taiwanese patients. The observed similarity in the occurrence of rare mutations among Nepalese, Chinese and Taiwanese nations, probably derived due to the common ancestor. Discovered mutations presumably prevent the completion of transcription and affect protein function. To better understand the mutational effects on protein structure, function and subsequently the impact on tumorogenesis, biochemical, cellular studies and Bioinformatics methods such as amino acid substitutions (AAS) prediction methods are suggested.
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INTRODUCTION

Colorectal Cancer Definition and Epidemiology

Colorectal cancer (CRC), also known as bowel cancer and colon cancer, is characterized by the development of malignant cells within large intestine or rectum. Based on the histologic features, gastrointestinal cancers can be subclassified into adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, small cell carcinoma, carcinoid tumor, or other (Ajani, et al., 2005). Colorectal adenocarcinoma has been reported with the highest incidence of malignancy in the GI tract (Kumar, et al., 2015). In most cases, colon adenocarcinoma starts as a benign adenomatous polyps, development of polyps can lead into advanced adenoma in five or more years (Longe, 2005; Markowitz & Bertagnolli, 2009). Determined CRC with high mortality is the second cause of death from cancer in the USA (Colussi, et al., 2013). The global colorectal cancer wide incidence demonstrates that, CRC is the second most common cancer among women and also third most common cancer in men (Armaghany, et al., 2012). Negligible government support in Nepal for management of cancer led to the establishment of all statistical analysis based on the preliminary reports of some teaching, cancer and general hospitals (Poudel, et al., 2016). Therefore Nepal categorized into groups with low incidence and mortality of colorectal cancer (Ghoncheh, et al., 2016).

Risk Factors

Genetic predisposition (20%) and environmental factors (80%) are common causes of CRC (Longe, 2005). Inherited risk factors include the determined genetic syndromes like Lynch syndrome, familial adenomatous polyposis, Juvenile polyposis syndrome and Peutz-Jegher syndrome (Rozen, et al., 2006). Environmental risk factors which have been reported to be associated the CRC include, high intake of animal fat, fried or charred red meat, low intake of grains, fruits and vegetables which contain fiber, minerals and vitamins (Rozen, et al., 2006). Other known risk factors are, occupation such as brewery workers, gender, age, obesity, physical activity, tobacco smoking, alcohol abuse, laxative usage, iron supplements and hemochromatosis, acromegaly, diabetes mellitus, cholecystectomy, radiation therapy for breast and ovarian cancer, human polyomavirus JC virus and inflammatory bowel disease (Rozen, et al., 2006).

Chronic inflammatory bowel disease (IBD) increases the risk of colorectal cancer by increases of epithelial cell turnover in the GI tract, and composed of two main disorders known as ulcerative colitis and Crohn’s colitis (Rozen, et al., 2006).

Clinical Features and Staging

The CRC symptoms are correlated to, anatomical site of tumors in large bowl, impact on the peripheral organs and stage of cancer (Longe, 2005; Mohan, 2010). Most important clinical symptoms of CRC are, occult bleeding (melaena), change in bowel habits, loss of weight (cachexia), loss of appetite (anorexia), fatigue and abdominal discomfort (Mohan, 2010). A cancer staging system is utilized for determining treatment process and prognosis, CRC staging consists of four different levels based on the following criteria:
Stage I: The tumor confined to the epithelium without affecting the muscle layer.
Stage II: The tumor invaded and passed all layers of the colon and probably interact with local tissue.
Stage III: Regional lymph node involved without spreading to another part of the body.
Stage IV: Cancer spread to distant organs or tissues (metastasis) (Longe, 2005).

**Pathogenesis**

The CRC develops through a multistep process consisting a series of histological, morphological and genetic events. Histological and morphological changes start with transformation of normal colonic epithelium to adenoma and finally adenocarcinoma. Accumulation of genetic and epigenetic alterations in the genome contributes to induction of neoplastic transformation of healthy epithelium (Bardhan & Liu, 2013).

Inherited and familial forms associate to different germline mutations, but sporadic derives from somatic mutations. Estimated numbers are 10% inherited, 25% familial and approximately 70% sporadic of all CRC cases (Haigis, 2013).

Three different genomic instability pathways are defined for sporadic and inherited CRC carcinogenesis: chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP) pathways (Haigis, 2013).

**Genomic Instability Pathways**

*Chromosomal instability pathway (CIN)*; estimated approximately 85% of colorectal cancer derived from Chromosomal instability (Beauchemin & Huot, 2010). Furthermore Chromosomal instability (CIN) detected in 65%–70% of sporadic colorectal cancers (Pino & Chung, 2010). Evidences proved chromosome instability occurs in the earliest stage of CRC tumorigenesis to late stage (stage I-IV) continuously (Edler & Kitsos, 2005). Chromosome segregation defects, telomere dysfunction, DNA damage response inactivity and loss of heterozygosity (LOH) are main mechanisms which induce chromosomal instability (Pino & Chung, 2010). The genomic alterations are caused by chromosomal instability capable to activate proto-oncogenes like KRAS and COX-2 and also able to inactivate tumor suppressor genes such as APC, P53 and loss of heterozygosity for the long arm of chromosome 18 (18q LOH) (Armaghany, et al., 2012).

*Microsatellite instability (MSI)*; MSI arises from inactivation of DNA mismatch repair (MMR) genes by, mutation (germline and somatic mutation) or transcriptional silencing. Most important involved genes in MMR are MLH1, MSH2, MSH6 and PMS2. Approximately 15% of all CRC cases represents microsatellite instability, especially in the second stage of pathogenesis (Sinicrope & Sargent, 2012). A large part of sporadic CRC, derived from epigenetic silencing, a prominent example of this process is the MLH1 gene. Hypermethylation of the MLH1 gene in the promoter region, repress the gene expression and consequently induce microsatellite instability. During the pathogenesis, MMR deficiency can empower the cancer cells to activate oncogenes like BRAF and cyclin D1 or inactivate the tumor suppressor genes such as TGF-βRII and BAX (Colussi, et al., 2013; Hong, et al., 2010).
CpG island methylator phenotype (CIMP); CIMP detected in approximately 20-30% of all CRC cases. Majority of protein-encoding genes, comprise CpG islands in promoter regions. Improper hypermethylation of CpG island in promoter regions can repress the genes which are involved in the cell cycle, programmed cell death, angiogenesis, DNA repair, invasion and adhesion. The high occurrence of CpG island methylation in a tumor is termed CpG island methylator phenotype. CIMP pathway recognized in a large percentage of sporadic microsatellite unstable CRC patients (Colussi, et al., 2013; Curtin, et al., 2011).

DNA hypermethylated gene sequences are used as tumor biomarkers in sporadic CRC cases for cancer assessment, diagnosis, molecular staging (CRC subtype) and prognosis. The abundant number of these biomarkers, allows for designing, gene panels to sporadic CRC cases (Hong, et al., 2010). Different panels constructed for CIMP phenotype defining have been defined by several research groups. The results of the panels are slightly different, except from the BRAF V600E mutation which were found in a large percentage of CRC cases (Curtin, et al., 2011).

**Tumorigenic Genes and Induction of CRC**

Colorectal cancer derives from both genetic and epigenetic alterations of DNA, genetic mutation and DNA methylation are a common phenomenon in CRC. APC gene alteration plays a critical role in CRC pathogenesis and both mutation and methylation are recognized as reasons of APC inactivation. Another most common mutations in colorectal cancer occur in, KRAS and BRAF genes (Kuipers, et al., 2015). Remarkable methylated genes which detected in CRC, including MLH1, MGMT and CDKN2A (Nazemalhosseini Mojarad, et al., 2013).

**Known Genetic Alterations in CRC**

**APC Gene;** the adenomatous polyposis coli (APC) is a tumor suppressor gene and located on chromosome 5q21-q22. The gene composed of 15 exons and contains 8538 bp, open reading frame encodes a 2,843 amino acid protein. The exon 15 possess, more than 75% of coding sequences. Approximately two-thirds of all APC mutations are detected in the mutation cluster region (codons 1250 to 1464) in the 5’ part of exon 15 (Bunz, 2016; Liz Leoz, et al., 2015). The most common germline (18%) and sporadic (7%) mutation in the APC gene is 5-bp (AAAGA) deletion at codon 1309 (AAA GAA AAG ATT → AAA GAT T), which leads to a truncated protein via frameshift mutation (Kashfi, et al., 2014; Bast Jr, et al., 2017).

Genetic change of APC firstly founded in familial adenomatous polyposis syndrome (germline mutation) then, detected in sporadic-type of colorectal cancer as well (somatic mutation). Furthermore, recently methylation has been detected as an alternative mechanism for APC inactivation in CRC cases. The APC gene product is an integral component of the β-catenin destruction complex in the cytoplasm. In normal cells this destruction complex degrade the β-catenin and prevent of β-catenin mediated cell adhesion and transcriptional activity. In APC mutation status, APC deficiency disintegrates β-catenin destruction complex, therefore, β-catenin levels increase and translucent in the nucleus and recruits the transcription factors. APC mutations are early events during the CRC pathogenesis. Also the
wnt/wingless signaling pathway is able to induce β-catenin level enhancement by rupture of the β-catenin destruction complex (Sepulveda & Lynch, 2013).

**BRAF Gene;** BRAF is a proto-oncogene, located on chromosome 7q34 and composed of 18 exons. In colorectal cancer most of the activating BRAF mutations occur in exon 15 at codon 600. The T-to-A base transversion in codon 600 (ACA GTG AAA → ACA GAG AAA) leads to the substitution of Val by Glu at the amino acid level (Rodrigo, 2016). The normal BRAF encoded protein is a serine/threonine protein kinase and belong to the RAF kinase family. The BRAF is a part of MAPK/ERK (RAS/RAF/MEK/ERK) pathway which usually responds to growth factors and cytokines, involved in the transduction of mitogenic signals from the cell membrane to the nucleus. During the signal transduction critical binding occurs between B-Raf and Ras proteins. This interaction plays a regulatory role in the signal transduction process. The BRAF mutation activates the MAPK/ERK pathway constantly and cause abnormal cellular growth, invasion and metastasis (Thiel & Ristimäki, 2013).

**KRAS Gene;** KRAS gene is a proto-oncogene and two structurally distinct KRAS (KRAS-1 and KRAS-2) exist. Just KRAS-2 represents functional activity and are located on chromosome 12q12.1. The KRAS-2 composed of 6 exons and consist of 4.5 kb, high percent of mutations (70%) occur at codon 12 in exon 2. In the colorectal cancer most frequent KRAS mutation is G-to-A base transversion (GCT GTT GGC → GCT GAT GGC) which leads to the substitution of Gly by Asp at the amino acid level (Kerr & Johnson, 2017). The normal KRAS gene product, K-Ras protein, localized at the cytoplasmic side of the cell membrane. Ligand-binding receptor tyrosine kinase capable to activates the K-Ras protein. Stimulated K-Ras protein performs as signal transducer and capable to regulate transcription factor activation, consequently K-Ras affect cell survival, differentiation, cytokine production, chemotaxis and phagocytosis. The K-Ras protein like the other members of Ras superfamily contains, intrinsic GTP hydrolyzing activity (GTPase). This ability enables K-Ras proteins to cycle between inactive (GDP-bound) and active (GTP-bound) condition. The highest rate of mutations among of the Ras family members occurs in KRAS gene and impact GTPase activity, thereby allows KRAS to remain active in signaling. Mutations in the KRAS gene is an early event in the CRC tumorigenesis process (Kim, 2009).

**Epigenetic Alterations in CRC**

In addition to direct gene mutations such as APC, BRAF and KRAS, which explained above, epigenetic alterations like DNA hypermethylation of promoters-associated CpG islands, occur commonly in colorectal cancer and induce gene silencing. Multiple genes have been reported to be silenced in colorectal cancer, including MLH1, MGMT and CDKN2A. Hypermethylation in the 5 CpG island of MLH1 take place at high frequency in sporadic primary colorectal cancers with microsatellite instability (Gearhart & Ahuja, 2011).

**MLH1 Gene (MutL Homolog 1);** the MLH1 is a tumor suppressor gene, located on chromosome 3 and encodes protein MLH1. MLH1 encodes a protein that is a part of post-replicative DNA mismatch repair mechanism (MMR) and plays a significant role in genome stability by microsatellite repeats preservation. Two different types of MLH1 gene alterations
occur, mutation and methylation in the CRC. Inherited MLH1 gene mutation (germline mutation) increases the risk for hereditary nonpolyposis colorectal cancer. In the sporadic form of CRC, epigenetic alteration such as hypermethylation of the MLH1 gene detected. Most often hypermethylation take place on the CpG island in the promoter of the MLH1 gene (McManus & Mitchell, 2014).

MGMT Gene (O\textsuperscript{6}-methylguanine DNA methyltransferase); MGMT gene is a tumor suppressor gene, located on chromosome 10 and encodes a DNA repair protein, O\textsuperscript{6}-methylguanine DNA methyltransferase, which removes alkyl adducts from O\textsuperscript{6} position of guanine and O\textsuperscript{4} position of thymine. Active MGMT protects normal cells against carcinogens, also protects cancer cells from chemotherapeutic alkylating agents such as dacarbazine (DTIC) or temozolomide (TMZ). The promoter methylation regularize the MGMT transcription negatively and reduce gene expression. The hypermethylation of the MGMT promoter, transcriptional silencing and the consequent elevation of G to A transition mutation commonly occurs in human CRC (Inno, et al., 2014; Van Meir, 2009).

CDKN2A (cyclin-dependent kinase inhibitor 2A); CDKN2A is a tumor suppressor gene, located on chromosome 2 and encodes CDKN2A protein which acts as inhibitor of CDK4 and 6. Therefore CDKN2A performs as a tumor suppressor gene and prevents tumorigenesis by induction of cell growth arrest and senescence (Hershey, 2009). CDKN2A mutations are detected in different kinds of cancer, but the frequency of CDKN2A mutation is very low in colorectal cancer (Trzeciak, et al., 2001). Conversely, CDKN2A promoter methylation occur commonly in colorectal cancer and leading to gene silencing (Xing, et al., 2013).

**Aim**

The main aim of this project was to evaluate if common or rare mutations were present in the genes APC, BRAF and KRAS in colorectal cancer samples from Nepalese patients. Another aim was to evaluate the gene expression level of MLH1, MGMT and CDKN2A in this material, and to evaluate any correlation between mutation status, expression level and clinicopathological features.

The relevance between tumor suppressor silencing and proto-oncogene mutations have already been studied in colorectal cancer patients. Positive association detected among MLH1 expression and BRAF mutation, also MGMT expression and KRAS mutation in Dutch patients (Vogel, et al., 2009). Moreover inverse association discovered between CDKN2A expression, KRAS and BRAF mutations in the American CRC patients (Shima, et al., 2010).
MATERIAL AND METHODS

Patient Specimens, DNA and mRNA isolation

The tumor tissues of 43 CRC patients were previously collected, stored, fixed in formalin, embedded in paraffin (FFPE) and evaluated by pathologists at the Tribhuvan University Teaching Hospital in Nepal. According to the three-tier classification system, cancer cases were divided in three groups, low grade, intermediate grade and high-grade. In Gothenburg University genomic DNA and total mRNA of samples were previously extracted by standard procedures from FFPE cancer samples, then mRNA was transcribed into complementary DNA (cDNA). DNA concentration was measured using the Nanodrop spectrophotometer. The results of DNA concentration measurement recorded between 0.9-210 ng/µl. The extracted DNA and cDNA were stored at -20°C for further analysis.

Mutations Analysis of \textit{APC}, \textit{BRAF} and \textit{KRAS}

Mutation analysis launched with amplification of predefined sequences of the \textit{APC} exon 15 codons 1260-1410, \textit{BRAF} exon 15 codons 590-632 and \textit{KRAS} exon 2 codons 5-25 by the hot-start polymerase chain reaction (PCR) method. PCR amplification was accomplished in a 20µl volume containing, 0.125µl AmpliTaq Gold DNA Polymerase, 2.5µl 10X Gold Buffer (AmpliTaq Gold DNA Polymerase with Buffer II and MgCl2 kit, Applied Biosystems), 2.5µl 2mM dNTP Mix, 1.5µl 25mM MgCl2, 1µl 10µM primer F, 1µl 10µM primer R, 10.4µl nuclease free water and 1µl DNA template.

Three frequently published primer pairs were selected for amplification of target (APC, BRAF and KRAS) genes. Primer set for APC were designed especially for codon 1309 and peripheral codons, BRAF primers designed for codon 600 and flanking sequences and KRAS primers were designed for codon 12 and adjacent codons (see Table 1.) (Suzuki, et al., 2015, Lasota, et al., 2014 and Akiyoshi, et al., 2013).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’→3’)</th>
<th>Reverse Primer (5’→3’)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>AGACTTATTGTAGAAGATAC</td>
<td>ATGGTTCCTCTGAACGGA</td>
<td>450</td>
</tr>
<tr>
<td>BRAF</td>
<td>TCTTCATGAAGACCTCACAG</td>
<td>AGCCTCAATTTCTACCACCC</td>
<td>127</td>
</tr>
<tr>
<td>KRAS</td>
<td>TTATAAGGCCGTGAAATGACTGAA</td>
<td>TGAATTAGCTGTATCGTCAAGGCAC</td>
<td>89</td>
</tr>
</tbody>
</table>

Following the optimized thermocycling conditions for different genes, three various PCR was run according to the Table 2.
Table 2. Thermocycler PCR running conditions for APC, BRAF and KRAS genes.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature, °C</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>94</td>
<td>2min</td>
<td>1X</td>
</tr>
<tr>
<td>BRAF</td>
<td>94</td>
<td>2min</td>
<td>1X</td>
</tr>
<tr>
<td>KRAS</td>
<td>94</td>
<td>2min</td>
<td>1X</td>
</tr>
<tr>
<td>Denaturation/enzyme activation</td>
<td>94</td>
<td>2min</td>
<td>1X</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30sec</td>
<td>40X</td>
</tr>
<tr>
<td>Annealing</td>
<td>52</td>
<td>1min</td>
<td>40X</td>
</tr>
<tr>
<td>Extension</td>
<td>70</td>
<td>30sec</td>
<td>40X</td>
</tr>
<tr>
<td>Final extension</td>
<td>70</td>
<td>7min</td>
<td>40X</td>
</tr>
</tbody>
</table>

In the next step, to control the accuracy and quality of amplified sequences, PCR products were subsequently examined on 1% agarose gel with plus GelRed and run at 100V for 30min (see Figure 1).

![Figure 1. Analyzing of APC (1), BRAF (2) and KRAS (3) genes PCR products on agarose gel. The polymerase chain reaction (PCR) was run using the extracted DNA and nuclease-free water for negative control. The PCR products were then resolved on 1% agarose gel along with GeneRuler 100bp ladder and stained with GelRed.](image)

The PCR products were purified using the Wizard SV Gel and PCR Clean- Up System kit (Promega), and concentration was measured using the Nanodrop spectrophotometer. Recorded measurements were between 67-72.9ng/µl for 4 samples of APC, 70.9-1079ng/µl for 23 samples of BRAF and 62.6-101.2ng/µl for 23 samples of KRAS. All 50 purified DNA samples with only forward primer were delivered to GATC Biotech company in Germany and sequenced via the dideoxy chain termination method of Sanger.

**Expression Analysis of MLH1, MGMT and CDKN2A**

Real-time quantitative polymerase chain reaction (qPCR) was used to evaluate the mRNA levels of MLH1, MGMT and CDKN2A in the samples where total mRNA had been reverse transcribed to cDNA. The qPCR was performed in a 5µl reaction mixture, containing 2.5µl TaqMan Fast Universal PCR Master Mix (2X), no AmpErase UNG (Applied Biosystems), 0.25µl Taqman gene expression assays probes (Applied Biosystems, GAPDH; Hs02758991,
MLH1; Hs00979919, MGMT; Hs01037698, CDKN2A; Hs00923894), 2µl cDNA and 0.25µl nuclease free water. In the following, thermal cycling parameters which designed especially for 7900HT fast system utilized in PikoReal real time PCR System. Among the 43 evaluated samples (the normal and adjacent tumor tissue samples are paired) just in 10 samples (23.2%), reference gene (GAPDH) has been detectable and the high Ct ratio was acquired. (see Table 4). The expression rate of the MLH1, MGMT and CDKN2A were determined by the comparative method (Livak's 2-ΔΔCt equation) in relation to the rate of a housekeeping gene GAPDH.

Table 3. Thermal cycling parameters for qPCR.

<table>
<thead>
<tr>
<th>Initial Step</th>
<th>PCR (40 Cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denature</td>
<td>Melt</td>
</tr>
<tr>
<td>Hold</td>
<td>Anneal/Extend</td>
</tr>
<tr>
<td>20sec, 95°C</td>
<td>1sec, 95°C</td>
</tr>
<tr>
<td></td>
<td>20sec, 60°C</td>
</tr>
</tbody>
</table>

Statistical Analysis

The IBM SPSS program version 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The clinicopathological features such as: age, gender, tumor location, gene mutation and gene expression level were analyzed. Furthermore Fisher’s exact test was used to determin the correlation between mutation-mutation status, mutation-downregulation and the association of mutation and downregulation- clinicopathological features. A p value <0.05 was considered significant.

Ethics

Access to the sample information provided by Professor Katarina Ejeskär, University of Skövde. The protocol of this clinical program designed agrees with the principles of the EORTC and the EEC Note for Guidance: Good Clinical Practice for Trials on Medicinal Products in the European Community. Thereby, all procedures were conformed with the World Medical Association Declaration of Helsinki 2013.
RESULTS

Mutation Analysis

In this study, material from 20 patients out of the 43 patients were excluded due to the incomplete sample information such as age, gender, clinicopathological report, thus the 23 remaining patients with colorectal cancer, which had been clinically and laboratory confirmed, were examined. The patients consists of 30.5% female and 69.5% male aged between 30-75 years. Collected data also indicate a higher incidence of colon cancer (69%) than rectal cancer (31%). A remarkable percentage (30%) of patients presented high grade cancer phenotype and the remaining patients displayed 22% intermediate grade and 48% low grade cancer.

APC Mutation Analysis

Among the 23 prepared PCR products for the APC sequencing, only four samples were shown bright band and correct molecular weight (450 bp) on the agarose gel. For sequencing only these four samples were used, results revealed no mutation in codon 1309 of APC. Further examination on codons 1301, 1354, 1357, 1365, 1367, 1389 and 1398 uncovered a new notable transversion heterozygous mutation in codon 1365 (AGT GGT GCT → AGT GCT GCT) in three out of four patients. Substitutions of Glycine residues by Alanine is the result of this mutation (see Figure 2).

![Figure 2](image)

**Figure 2.** Identification of a novel mutation of the APC gene. Direct sequencing revealed a heterozygous probably nonsense mutation (B) in the exon 15 codon 1365 and (A) control DNA carried the wild type sequence.

BRAF mutation analysis

BRAF direct sequencing analysis showed that one of 23 samples contained a BRAF pathogenic mutation in codon 600, while the others did not show any mutations (see Figure 3). As previous explanation, in this mutation T was replaced by A in codon 600 (ACA GGT AAA → ACA GAG AAA) and called V600E mutation (see Figure 3). Codons 602, 605, 607, 612 and 616 were examined too and all were mutation-negative. The codon 600 mutation detection frequency was 4.3% in the 23 patients.
Figure 3. Wild-type (A) and mutant (B) sequence chromatograms of BRAF exon 15 (portion). Both the wild type and the mutant DNA were sequenced on the forward strand. The BRAF mutation-positive sample (mutant) shows the T-to-A transversion at codon 600.

**KRAS mutation analysis**

No KRAS mutations in codon 12 were detected in 22 out of 23 cases, only one sample consisted of a transition mutation in codon 12, the incidence of this mutation was 4.3%. According to the prior description, in this transition mutation G replaced by A (GCT GGT GGC → GCT GAT GGC, p. G12D) (see Figure 4).

Figure 4. KRAS mutation detection, a comparison of the sequence chromatogram between wild type (A) and mutant (B) KRAS exon 2, showing a transition mutation at codon 12.

For more investigation, codons 13, 14 and 15 assayed and a novel mutation was discovered. Deciphered transversion mutation occurred in codon 15, a G substituted by T (GTA GGC AAG → GTA TGC AAG, p. G15C) (see Figure 5).
Figure 5. Electropherogram for KRAS mutant. A novel transversion mutation in exon 2 codon 15 of KRAS gene was identified in 26% of Nepalese CRC patients. The mutation-positive (B) sample shows the G-to-T substitution at codon 15 compared to wild-type (A).

This mutation was detected in 6 out of 23 patients and thus the frequency of mutation was 26%.

Expression Analysis

The expression assessment of MLH1, MGMT, CDKN2A and GAPDH genes was achieved by qPCR using the TaqMan technology. The cDNA samples were evaluated via specific designed primers in four distinct reactions, PCR product sizes were verified by gel electrophoresis (see Figure 6).

Figure 6. Agarose gel electrophoresis of qPCR products (MLH1, MGMT, CDKN2A and GAPDH) to accuracy verification.

As shown in Figure 6, target sequences copied in exact size by primers except CDKN2A which was no amplified and MGMT with larger amplification products. The expected amplification sizes of MLH1, MGMT, CDKN2A and GAPDH are 104bp, 71bp, 115bp and 93bp respectively. Amplification charts of qPCR in three different reactions for GAPDH, MLH1 and MGMT represented in Figure 7.

Figure 7. Amplification chart of qPCR for three reactions, extracted cDNA from human neuroblastoma cell line utilized as positive control.

Satisfactory results were achieved in 23.2% of samples. Probably due to gene silencing, in 100% of acceptable cases, CDKN2A no gene expression was detected. MLH1 and MGMT expression assessment results were similar, likely owing to gene silencing as well, in 80%
cases, both gene expressions were undetermined (see Table 4). The qPCR data analysis showed, that MLH1 gene expression levels in cancerous samples was reduced compared to mucosa in the same patient, and that the MGMT gene expression levels was increased in one sample and decreased in one sample. Nonetheless, due to a small number of samples, statistical analysis was not applicable.

A summary of Ct values and data analysis of all 10 samples is provided by table 4.

Table 4. Outcomes of data analysis using the Livak’s 2-ΔΔCt method. The fold change in expression of the target gene relative to the internal control gene (GAPDH) was studied.

<table>
<thead>
<tr>
<th>Sample No</th>
<th>GAPDH Ct</th>
<th>GAPDH Ct</th>
<th>MLH1 Ct</th>
<th>MLH1 ΔCt</th>
<th>MLH1 ΔΔCt</th>
<th>Fold Change</th>
<th>MGMT Ct</th>
<th>MGMT ΔCt</th>
<th>MGMT ΔΔCt</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>30.47</td>
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●Mucosa and ●Tumor, samples Ct value analysis. ΔCt= Ct Target Gene - Ct Reference Gene, ΔΔCt (Cancer) = ΔCt Cancer - ΔCt Mucosa, Fold Change= 2^-ΔΔCt.

All data after collection were analyzed by SPSS software (version 21.0). The qualitative variables were analyzed by Chi-square and Fisher's exact test to find correlation between mutation, expression and stage of cancer. But due to the small size of the samples, statistical analysis was not applicable in the most of the cases. The statistical conclusions are as follows; 66.7% of intermediate grade cancer cases represented APC codon 1365 mutation (p-value= 0.120, comparison between codon 1365 mutation and cancer stages). In 50% of APC codon 1365 mutated cases MLH1 expression decreased and in 50% MLH1silencing detected (P-value=0.236, comparison between mutations and expressions). Also in 50% of APC mutated samples MGMT expression increased and in 50% MGMT silencing was discovered (P-value=0.104 , comparison between mutations and expressions). In 100% of KRAS codon 15 mutated cases MLH1 and MGMT gene silencing detected (p-value= 0.429 and 0.732, comparison between mutations and expressions).
DISCUSSION

The purpose of this study was to identify different mutations, assessment of gene expression level, identifying correlations between pathogenicity, mutations, gene expression and consequently, organizing for first time preliminary colorectal cancer biomarkers guideline especial for Nepalese patients.

The discovery of APC mutations in CRC was a great success to identify mutation carriers in clinical screening (Vasli, et al., 2007). Presymptomatic diagnosis of APC mutation by genetic tests lead to preventive colectomy in susceptible individuals (Vasli, et al., 2007). Furthermore, in cancer patients, APC mutation discovery performed as a basis for patient classification and designing effective treatment (Vasli, et al., 2007). The nonsense or frameshift mutations defines common types of APC mutations, which create truncated proteins. Different methods have been used for mutation identification in the APC gene (Vasli, et al., 2007). Direct sequencing analysis proved as an effective method for examination of APC coding, flanking and intronic regions (Leonard, 2007). Therefore, Kashfi and colleagues recently utilized direct sequencing method for detecting inherited APC mutation in the Iranian FAP families and reported 50% deletion rate at codons 1309 (Kashfi, et al., 2014). Also Chen and colleagues reported 14% deletion rate at codon 1309 in the Chines FAP families by using direct sequencing method too (Chen, et al., 2015). In addition, Nieuwenhuis & Vasen research conclusion demonstrated non essentiality of codon 1309 mutation for disease onset and severe polyposis (Nieuwenhuis & Vasen, 2007). In this study, a novel mutation was identified in the APC at codon 1365 (GGT → GCT) (Figure 2), similar mutation has been reported previously. Yuan and colleagues deciphered a nonsense mutation (GGT → GGC) at codon 1365 in CRC cases and reported 8% frequency of mutation in Chinese patients (Yuan, et al., 2001). In the USA, Inra J.A. and colleagues were able to demonstrate the high rate of APC mutations in the Asian and African compared to is European. Also Inra J.A. and colleagues showed a high incidence of CRC development in young age in Asian and African with strong polyps phenotype (Inra, et al., 2015). Furthermore, Kastritis and colleagues detected missense mutation (GGT → GAT) at codon 1365 in urothelial carcinomas but the prevalence of mutations in the Greeks has not been evaluated (Kastritis, et al., 2009). Due to the small size of the examined samples in this study, determining associations between phenotypes and APC mutation is impossible.

Regarding the magnitude impact of BRAF and KRAS mutations on the planning of treatment strategies and disease prognosis, consideration of the above genes were prioritized. Prior numerous investigations presented low prevalence (5-10%) of BRAF mutation in sporadic CRC cases (Dolatkhah, et al., 2015). In China, large-scale investigation performed by Li & Ma and 4.7-10% BRAF mutation incidence rate was recorded (Li & Ma, 2014). A study recently conducted in India by Jauhri and colleagues confirmed the low prevalence of BRAF mutation, mutation frequency reported around 7.1% in Indian CRC cases (Jauhri, et al., 2017). Similar research was accomplished in Singapore by Phua & colleagues and no mutations were detected in codon 600 among Singaporean cases (Phua, et al., 2015). In this study, we observed 4.3% codon 600 mutation frequency (Figure 3), due to the restricted
number of mutated cases, description of the correlation between mutations and clinicopathological features was impossible.

The KRAS, codon 12 mutation frequency in colorectal cancer has been reported in a range of 20 -50% (Edalat, et al., 2006). Hu and colleagues in China were determined KRAS codon 12 mutation incidence about 35% in CRC patients (Hu, et al., 2016). In Singapore Phua and colleagues represented KRAS codon 12 mutation occurrence around 25% in colorectal cancer cases (Phua, et al., 2015). Furthermore, in UK, Jones and colleagues reported 31.5 % incidence of KRAS codon 12 mutation among patients from England (Jones, et al., 2017). While in this study, mutation frequency of KRAS codon 12 was determined to 4.3% (Figure 4). The difference in the mutation frequency between prior research and this study can derived from different populations which was utilized for assessment. Also, perhaps difference obtained due to genetic or ethnic diversity, diet, environmental effects, social lifestyle and other factors that can be specific to the Nepalese people.

In this study a novel mutation in KRAS, codon 15 with a 14% incidence was discovered (GGC → TGC) (Figure 5). Similar mutation (GGC → GCC) have been reported for just one time in the world by Wang and colleagues in Taiwanese patients with a 32.1 % incidence. The Taiwanese research group demonstrated mutation at codon 15 of the KRAS reduced GTPase activity in the CRC. Also, this research team showed no correlation between codons 12 and 15 mutations (Wang, et al., 2003). Due to incomplete recorded clinical data, it was impossible to find the correlation between clinicopathological features and mutations.

The study of MLH1 expression at protein level has been carried out by a large number of researchers, but we performed an assessment of MLH1 expression at mRNA level in Nepalese CRC patients. A similar study has been conducted in Romania by Ioana & colleagues and proved MLH1 expression at mRNA level reduction in sporadic CRC (Ioana, et al., 2010). In Brazil, Leguisamo and colleagues have identified down-regulation of MLH1 at mRNA level in 31% of all sporadic CRC cases (Leguisamo, et al., 2017). Outcomes of mentioned researches confirmed the results of this study. In this study MLH1 expression was generally at an undetectable low level (Table 4), also reference gene (GAPDH) expression detected very low (Table 4). The obtained results probably declare poor quality of cDNA which provided via University of Gothenburg for further investigation.

Most of researches demonstrated down-regulation of MGMT at the protein level in the CRC. In Korea, Kim and colleagues observed loss of MGMT expression at the protein level in almost all sporadic cases with methylated alleles, inversely in sporadic cases without methylated allele a positive expression was identified (Kim, et al., 2003). In Brazil, Leguisamo and colleagues detected MGMT expression reduction at mRNA levels in 73% of sporadic cases (Leguisamo, et al., 2017). In the similar research in Brazil by Cordeiro and colleagues found MGMT down-regulation at mRNA level in all sporadic cases determined (Cordeiro, et al., 2012). The results of this study confirmed the conclusion of previous mentioned researches. Like MLH1, MGMT and GAPDH expression was generally at an undetectable low level (Table 4), and the low quality of the samples was raised.
Clinical evidences and observations indicated CDKN2A prognostic significance, however, this still remains in uncertainty. A recent study in Turkey by Yoruker and colleagues proved up-regulation at mRNA level of CDKN2A in 75% of CRC cases (Yoruker, et al., 2012). In Brazil, by Almeida and the colleagues CDKN2A methylation among cancer samples showed a 40% downregulation of expression (Almeida, et al., 2015). In the USA, Shima and colleagues in a large-scale research effort observed that CDKN2A expression loss at protein level was common in CDKN2A-methylated tumors (61%) (Shima, et al., 2011). In this research, we could not detect any CDKN2A expression in any of the samples tested and like the MLH1 and MGMT low quality of provided samples suspected.
ETHICAL ASPECTS AND IMPACT OF THE RESEARCH ON THE SOCIETY

For comprehensive molecular analyses, human tissue specimens were utilized. Due to the provision of samples in the form of FFPE, cell culture and supplying cell line was impossible and samples directly were used. As mentioned prior, the main purpose of this research was organizing for first time preliminary colorectal cancer biomarkers guideline for Nepalese patients to the screening and prognosis of treatment.

The results of our study and prior investigations have shown that researchers should consider some of the genetic differences in South-East Asia, because some rare mutations had already been found only in that part of the world.

The results of this research suggest, the necessity of more attention to rare mutations and the requirement of the parallel diagnostic program, especially for rare mutations beside the common mutation detection program for Nepalese CRC patients.
CONCLUSIONS

The mutations of APC, BRAF and KRAS are important events in colorectal carcinogenesis. Despite large-scale studies of APC, KRAS and BRAF mutations as a principle of therapeutic strategy determination, limited data from the frequency of these mutations exist for Nepalese people. In this study, we were able to discover unique novel mutations in Nepali’s CRC patients for the first time. Similar rare mutations in APC codon 1365 reported just one time in Chines CRC patients and KRAS codon 15 mutation only reported in Taiwanese patients.
**Future perspectives**

We have succeeded in identifying novel *APC* and *KRAS* mutations in a small number of samples. To confirm the accuracy of these findings, repetition of current experiment in a large number of samples is required and suggested. Certainly both, forward and reverse primers need to be used for sequencing.

Due to inadequate supplied information related to identified two novel mutations, the impact of these mutations in pathogenesis is almost unknown. To better understand the mutational effects on protein structure, function and subsequently the impact on tumorogenesis, biochemical, cellular studies and Bioinformatics methods such as amino acid substitutions (AAS) prediction methods are suggested.
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