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Spotting rs112445441 in Non-Hodgkin Lymphoma: Another Clue for the Context-Dependent Crosstalk between RAS-MAPK and PI3K Mediated Pathways

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ARS conceived and supervised the research. BNM and MSN conducted the experimental work, BNM, MSN and SIN wrote the article, MSC provided the samples for study.

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K-ras mutant isoform, MAPK pathway, SIFT analysis, Phosphatidylinositol-3-kinase (PI3K) mediated pathway, Ras/PI3K/PTEN/Akt/mTOR pathway, BRAF gene.

ABSTRACT

Phosphatidylinositol-3-kinase (PI3K) mediated or Ras/PI3K/PTEN/Akt/mTOR is one of the major effector pathways in the pathogenesis of Non-Hodgkin Lymphoma (NHL). Binding of growth factors/ mitogen/cytokine or interleukin to EGFR leads to Ras induced RAF-MAPK cascade activation. PTEN protein is involved in the negative regulation of PI3K pathway. Mutations in upstream kinases, growth factor receptors or intrinsic members of cascades can lead to induce or promote cancers. Number of somatic mutations in several genes, majority of which are involved in chromatin modification and transcriptional regulation, have been reported in NHL. G468R and G468A mutations in *BRAF* gene have been reported in NHL, BRAF is a member of RAS mediated MAPK pathway. In current study, hot spots of K*ras* gene were analysed in a 40 years old male patient, presented with NHL located in ascending colon with worst prognosis of disease. Through mutagenic PCR, codon 12 was analysed by creating a single nucleotide mismatch at the 3′-end of primers to produce a *Bst*NI recognition sequence at codon 12 while codon 13 was analysed by introducing *Hae*III recognition sequence. By using RFLP and sequencing, point mutation substituting the glycine (GGC) to aspartic acid (GAC) was observed at codon 13. The p.G13D or cDNA.230G>A/ g.5590G>A is previously recognized as rs112445441 and being reported for the first time in NHL. By in silico analysis, it is anticipated to be a diseases causing or pathogenic alteration by Mutation taster and SIFT analysis. Spotting the rs112445441 in NHL is supporting the idea of cross talk between RAS-ERK-MAPK and PI13K and this could be one of the factors behind the development of resistance to the current therapy and relapse in NHL. K ras mutant isoforms, being the negative predictors for prognosis, are vital to analyse before the start of adjuvant therapy. Further studies needed to confirm the functional aspects of rs112445441 and other variants involved in NHL pathogenesis.

INTRODUCTION

Non-Hodgkin lymphoma (NHL) makes up about 90% of all malignant lymphoma has become seventh most frequently occurring cancer (Ekstrom-Smedby, 2006; Qiao *et al*., 2014). The reported prevalence in Southeast Asian and Central/South American countries were 5.2 and 3%, respectively, as compared with just 0.3% in North American and European countries (Perry *et al*., 2016). In Pakistan, the reported age standardised incidence rate recorded in 1995, was 5.3/100,000 and 4.1/100,000 in males and females, respectively, which increased to 8.4/100,000 in males and 6.5/100,000 in females when recorded in 2002. People from the North western regions of the country, especially with low socio-economic condition and children were found to have greater risk of developing

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Abbreviations

EGF, epidermal growth factor; ERK, extracellular-signal-related kinase; GAP, GTPase-activating protein; GEF, guanine-nucleotide-exchange factor; Grb, growth-factor-receptor-bound protein; GAB, Grb2-associated binding partner; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKP, MAPK phosphatase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase;; PLCγ, phospholipase Cγ; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SFK, Src family kinase; SH, Src homology; TNFRSF9, Tumor necrosis factor receptor subfamily 9.

the disease (Bhurgri *et al*., 2005). It was ranked at 4th top most malignancy (both genders) in all age groups and mostly falls into intermediate to high grade category (CRCDM, 2011; Pervez, 2012). Accounting 80-85% of total NHL incidences, B-NHL is the most common type of NHL, followed by T-NHL. Use of hair dye, diets rich in fats, higher BMI, exposure to few chemicals and certain gene-environment interactions are responsible for such mutations (Bassig *et al*., 2012). Modification of histones (post-transcriptional) is crucial in germinal centre B cells. Genetic alterations in related genes can deregulate these modification leading to the enhanced methylation and reduced acetylation which are the key steps involved in the development of NHL (Morin *et al*., 2012). Based on the expression pattern of LMO2 and TNFRSF9 (tumor biomarker and tumor microenvironment marker respectively), a two-gene model was proposed in B-NHL (Alizadeh *et al*., 2000). Although, several types of therapies have been developed yet the molecular genetics of NHL is not very clear for all subtypes (Alizadeh *et al*., 2000; Guo *et al*., 2016). Besides the CD20 and members of PI3K pathway, other major genes observed to be altered in NHL are MLL2, BCL2, CARD11, CD79B, EZH2, IRF4, MEF2B, TP53, BTG1, BTG2, CREBBP, GNA13, SGK1, B2M, ETS1, FAT2, IRF4, FOXO1, STAT3, RAPGEF1, ABCA7, RNF213, MUC16, PIM1, COL4A2, EP300, SAMD9, PRKDC, HDAC7, FAS, CIITA, TMEM30A, KLHL6, MYD88, CD70, CD58, CD79B and CCND3. With truncating mutations, MLL2 was found to be frequently mutated tumor suppressor gene in NHL (Morin *et al*., 2012). Contribution of proto oncogene like BCL-1, BCL-2, BCL-6 and c-MYC have also been relevant for clinicians when treating NHL (Gaidano *et al*., 1995). A novel variant *TNFRSF13C has been identified in* the gene encoding human B cell-activating factor receptor in NHL (Rodig *et al*., 2005). Mutations in BRAF gene in NHL tumors have been reported by Lee *et al*. (2003). KRAS is a GTP- and GDP-binding protein, plays an important role in signal transduction. In the inactive state it is bound to GDP, in the active to GTP. Guanine nucleotide exchange factor (GEF) acts as a positive regulator by promoting dissociation of GDP, while GTPase activating protein (GAPS) acts as a negative regulator by promoting hydrolysis of GTP. Pi, inorganic phosphate (Watzinger and Lion, 1999). Usually release of GDP is regulated by the intracellular concentration of GTP. Mutations in K ras gene have been reported in various cancer types, i. e pancreatic, colorectal, lung adenocarcinomas (Barbacid 1990) and thyroid lymphoma which develops in autoimmune thyroiditis (Takakuwa *et al*., 2000). Codon 12, 13 and 61 have been found to be widely studied hotspots of the gene, found to be mutated in a wide range of cancers. Point mutations reported in other codons are in codon 11 (Hongyo *et al*., 1995), 12 (Murtaza *et al*., 2014), 13 (Moerkerk *et al*., 1994; Bazan *et al*., 2002), 15, 18 (Singer *et al*., 2003), 19 and 20 (Akagi *et al*., 2007; Naguib *et al*., 2011), 22 (Miyakura *et al*., 2002), 27, 30 (Wang *et al*., 2003), 31 (Murtaza *et al*., 2012), 61 (Enomoto *et al*., 1992), 117, 146 and 154 (Edkins *et al*., 2006; Teneriello *et al*., 1993; Neumann *et al*., 2009; Dogan *et al*., 2012). With advanced clinical stages of CRC, codon 13 mutations have been associated with lymph node metastasis (Moerkerk *et al*., 1994; Bazan *et al*., 2002).

Patients having metastatic CRC with K ras p.G13D mutation have found to have better prognosis when treated with cetuximab as compared to codon 12 mutant variant. The underlying mechanism is the similar behaviour of c.38G.A K ras as wild type K ras (Kumar *et al*., 2014; Chen *et al*., 2013). In a preclinical trial, codon 13 mutation showed sensitivity towards cetuximab in an *in vitro* LoVo cell line model (Kumar *et al*., 2014). CRC cells harboring p.G13D observed to be more sensitive to anti-EGFR treatment (Messner *et al*., 2012). Codon12 mutations in K ras represents an aggressive phenotype of tumour as compared to codon 13 mutations by altering the threshold level for apoptosis induction in CRC (Guerrero *et al*., 2000). Modelling of G13D onto the wild type K ras structure demonstrated that the side chain atoms of Asp13 face the opposite side of the P-loop, its 4 Å from Tyr32 (Lu *et al*., 2015) (Fig. 1).

MATERIALS AND METHODS

Patients and samples

A 42 year old male patient presented for the surgical resection of a growth located in ascending colon was the case for study. After the informed consent, a small piece of tumour tissue, its adjacent normal tissue (12 cm away from the tumour location), whole blood (5 ml) was taken. The tissue samples were immediately transferred to liquid nitrogen, and then stored at -80^{0}C until further processed. Genomic DNA from tissue samples was extracted using Puregene DNA extraction kit (Qiagen) and from blood the protocol established by Helms (1990) as followed.

Analysis of K ras hotspots

Through mutagenic PCR, codon 12 was analysed by creating a single nucleotide mismatch at the 3′-end of primers to produce a *Bst*NI (Thermo Fischer Scientific Cat. No. 0551) recognition sequence at codon 12. Following primers were used:

Forward: 5’ actgaatataaacttgtggtagttgga**c**ct 3’

Reverse: 5’ tcaaagaatggtcctgcacc 3’.

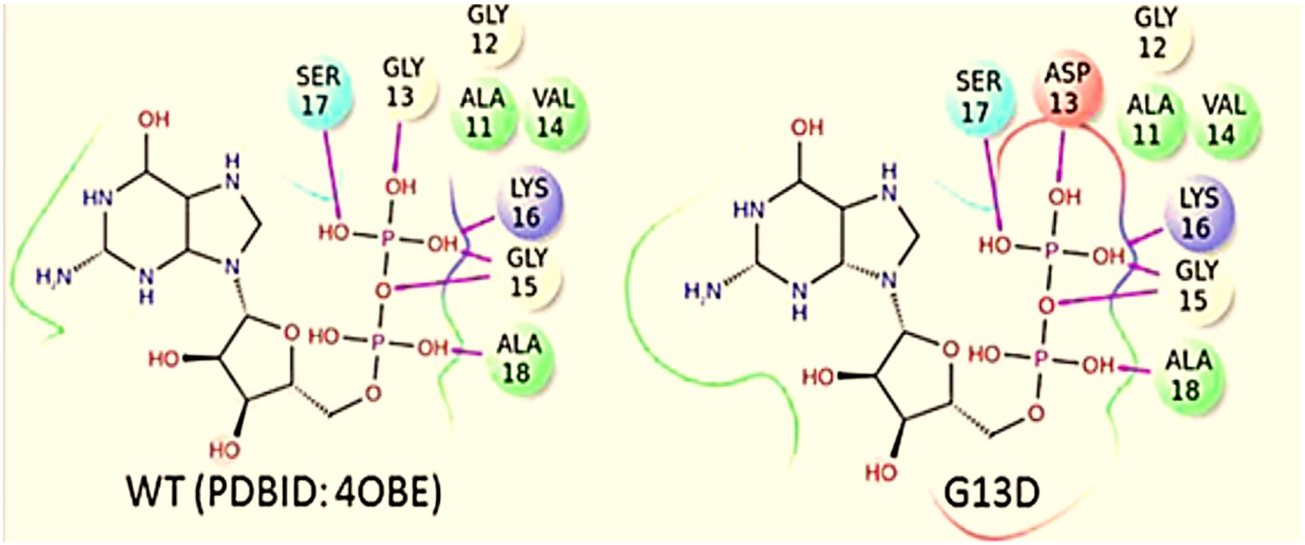

Fig. 1. Scheme of Hydrogen bonding between β-phosphate of GDP and backbone of P-loop (residues 13–17) in WT (left) and G13D (right) (Figure adapted from Lu *et al*., 2015). RAS family is direct or indirect regulator of complex signalling mechanism like, RAS-ERK-MAPK, PI13K, ral guanine nucleotide dissociation stimulator (RALGDS) and protein kinase C (PKC) etc. (Khan *et al*., 2018), hence involved in plethora of molecular activities associated with normal cellular functions.

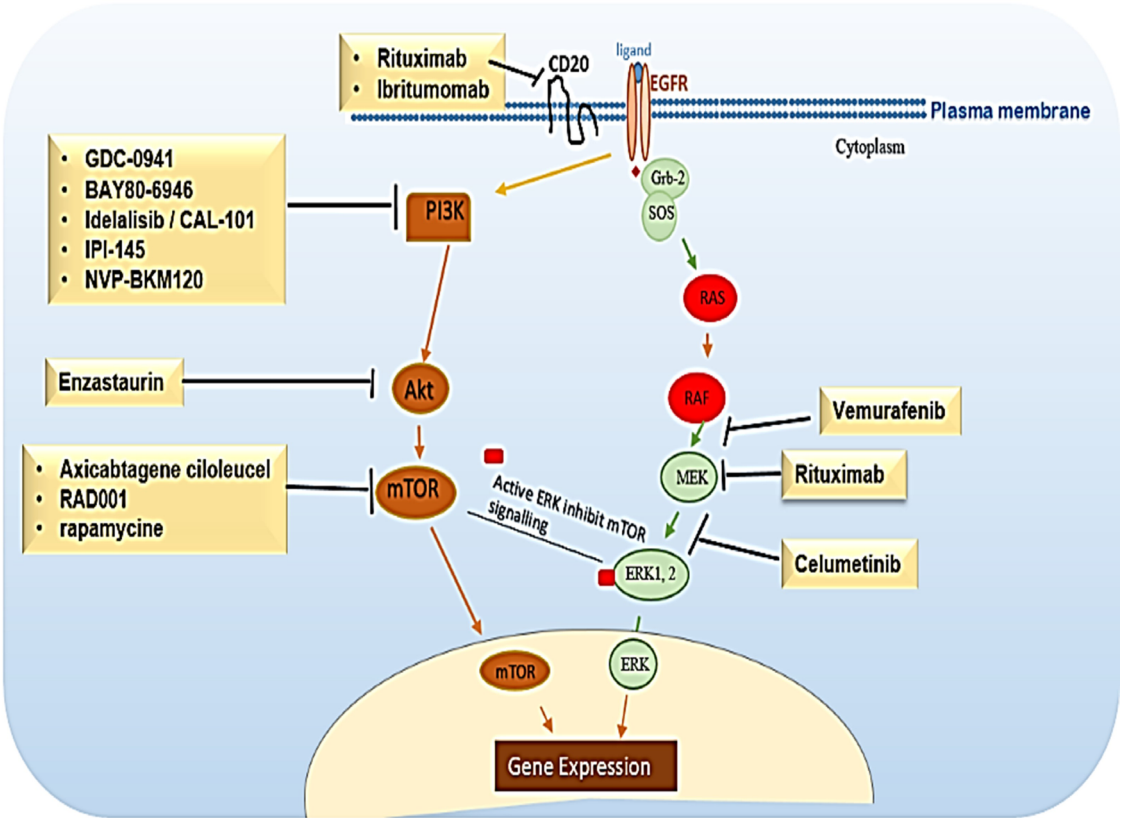

Fig. 2. A, Sequence alignment by BLAST; B, 12 % Acrylamide: bisacrylamide electrophoresis photograph showing DNA fragment containing codon 13 after restriction. Sample lanes 1-7 and 9, wild type; lane 8, NHL (heterozygous mutant); and lane M is 50 bp DNA marker (Fermentas); C, Sequencing shwoing GGC (glycine) to GAC (aspartic acid).

Forward primer carried a mismatched nucleotide (underlined). The cleavage site would be absent in the case of mutated codon 12. In PCR, 50 ng of gDNA with 0.25 units of Taq and 20 pmoles of each primer was amplified at 60°C annealing temperature. Approximately, 250 ng DNA was restricted with 20 units of *BstN*1 by overnight incubation at 37°C (Prall and Ostwald, 2007). Codon 13 was analysed by following the protocol established by Hatzaki *et al*. (2001). An *Hae*III recognition sequence was introduced in the PCR-amplified wild-type alleles through a mutagenic PCR. Forward primer with nucleotide sequence 5′ gtactggtggagtatttgatagtgtattaa 3’ and reverse with 5′ gtatcgtcaagg’cactcttgcctagg 3′ were used. For reaction, 50 ng of gDNA with 0.25 units of Taq and 20 picomoles of each primer was amplified at 50 °C annealing temperature. Approximately 250 ng DNA was restricted with 20 units of *Hae*III (Thermo Fischer ER# 0151) by an overnight incubation at 37°C.

Protocol established by Sills *et al*. (1999) was followed to analyse the codon 61. The forward primer with nucleotide sequence 5’ gacatcttagacacagcagtt ‘3 and reverse with 5’ tagccataggtggctcacct ‘3 were used for the PCR. For the reaction, 50 ng of gDNA with 0.1 unit of *Taq* and 20 picomoles of each primer was amplified at 59°C annealing temperature. The normal sequence of codon 61 is CAA and there are restriction sites for *Xba*I (Thermo Fischer #ER0681), *MSE*I (Fermentas Life Sciences, ER# 09825), and *Taq*I (Fermentas Life Sciences, ER# 0671) enzyme were created by the presence of A to T, C to A or A to G, mutation respectively, in the first or second base of codon 61. The presence of mutations was finally confirmed by DNA sequencing by using capillary electrophoresis-based sequencing services (Applied Biosystems (ABI) 3730xl DNA Analyser). Presence of mutations was analysed by SeqMan sequence assembly software. For analysing the mutations bioinformatics tools like Blast alignment, Sorting Intolerant from Tolerant (SIFT) (Ng and Henikoff, 2003) Mutation Taster and ProMod3 (Guex *et al*., 2009) were used.

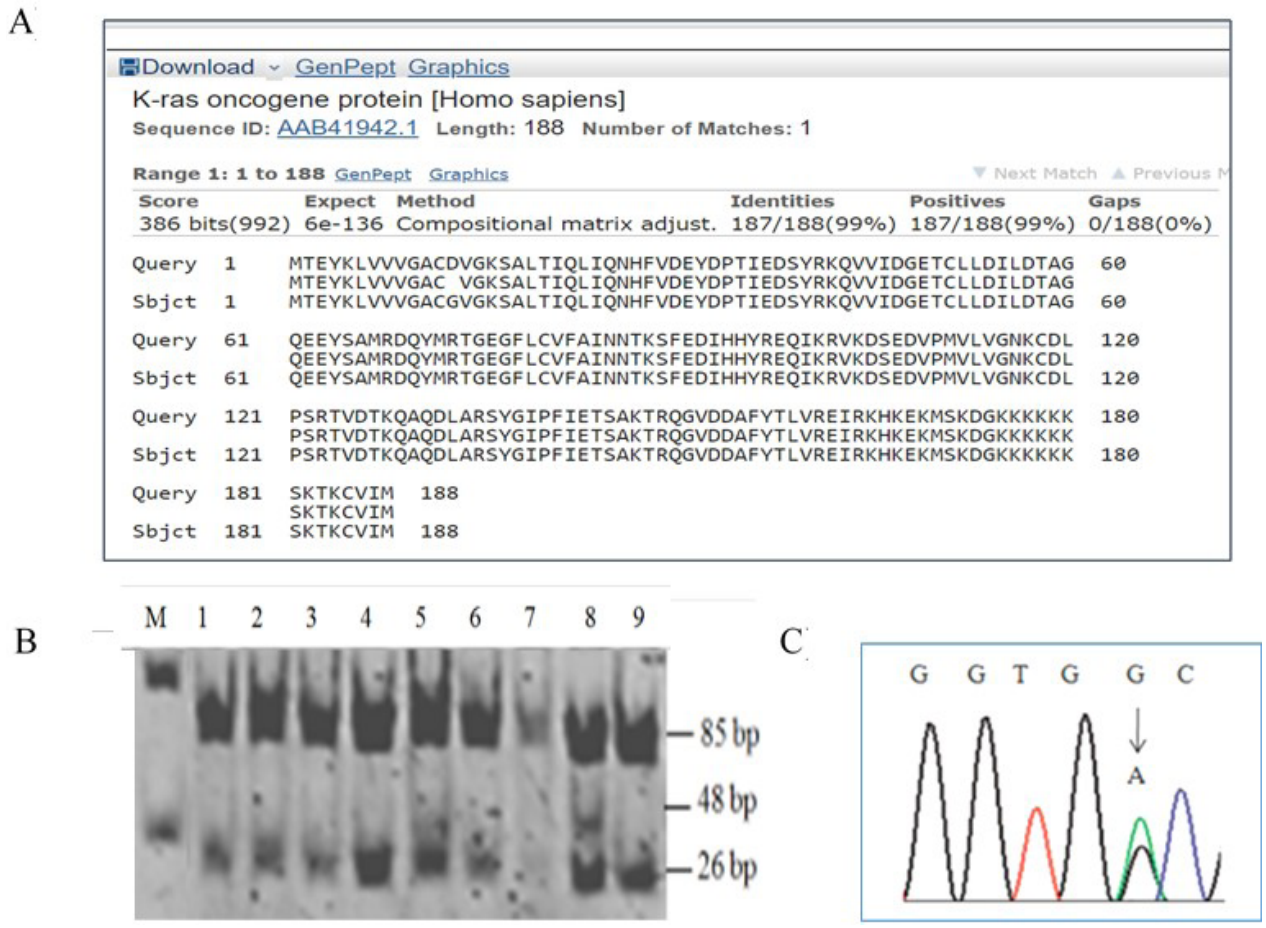

Fig. 3. Context-dependent crosstalk between RAS-MAPK and PI3K mediated pathways. (Figure adapted).

RESULTS

The patient under study was going through the routine surgical resection for NHL confirmed by histopathological examination of biopsy. Tumour was negative for any p53 mutations. K ras hotspots i.e codon 12, 13 and 61 were analysed. For codon 12, in case of wild type allele, *Bst*NI digestion of codon 12 should result in 2 bands of 29 and 128 bp, whereas mutant should remain uncut product of 157 bp. After *Hae*III restriction, wild-type codon 13 should result in 3 bands (additional fragment due to an internal *Hae*III recognition site) of 85, 48, and 26 bp, but mutant allele would be digested into 2 bands of 85 and 74 bp. For codon 61, CTA, AAA and CGA mutations were analysed by *Xba*I, *MSE*I and *Taq*I digestion, respectively. Wild type codon 61 should not be cut by these enzymes. Herein, no mutation was found at codon 12 and 61. Codon 13 was observed to have alteration (Fig. 2A), which was subsequently confirmed by sequencing and alignment (Fig. 2B and 2C), a G to A transition at second base of the codon (p.G13D), substituting glycine (GGC) to aspartic acid (GAC). p. G13D or cDNA.230G>A/ g.5590G>A/ rs112445441 is an already a recognized mutant variant. According to our knowledge based on published reports, this variant has never been reported in NHL before. K RAS wild type, K RAS p.G13 Models were constructed using ProMod3 based on template–target alignment. p. G13D scored 94 and predicted to be a disease causing or pathogenic alteration by Mutation taster. Based on Bayes classification, Mutation Taster counts the score (0.0 to 215) from an amino acid substitution matrix that depends on the difference between the physicochemical properties of amino acids involved. According to SIFT analysis p. G13D is DAMAGING with the median conservation 3.37. SIFT is based on the changes in sequence homology by substitutions and low confidence predictions with Median conservation above 3.25 will be declared as damaging.

DISCUSSION

The role of PI3K pathway is well understood in NHL pathogenesis. To reduce the overexpression of the pathway, number of inhibitors including RAD001 targeting mTOR, idelalisib or CAL-101 (GS-1101) targeting p110δ, IPI-145 targeting p110γ/δ, NVP-BKM120, GDC-0941 and BAY80-6946 targeting PI3K have been developed (Fang *et al*. 2014). Rituximab, a monoclonal antibody targeting CD20, in combination with other agents like vincristine, doxorubicin, cyclophosphamide and prednisone has proven to be a therapy of choice for NHL (Coiffier *et al*., 2010) but the development of resistance against the hyper activated pathways is also evident. Some of new agents are in trials and showing promise. Axicabtagene ciloleucel or Yescarta is also another option approved by FDA (FDA, 2018). Despite the presence of variety of these therapeutic agents, poor prognosis is still a challenge especially in relapsed cases and with salvage regimens (Chao, 2013; Cheson, 2014). The possible involvement of RAS–RAF-MAP kinase pathway cannot be ignored. In a combined therapy for treating KRAS mutant CRC, it has been observed that BKM120, an inhibitor of PI3K enhances the efficacy of cetuximab. Both pathways can activate or

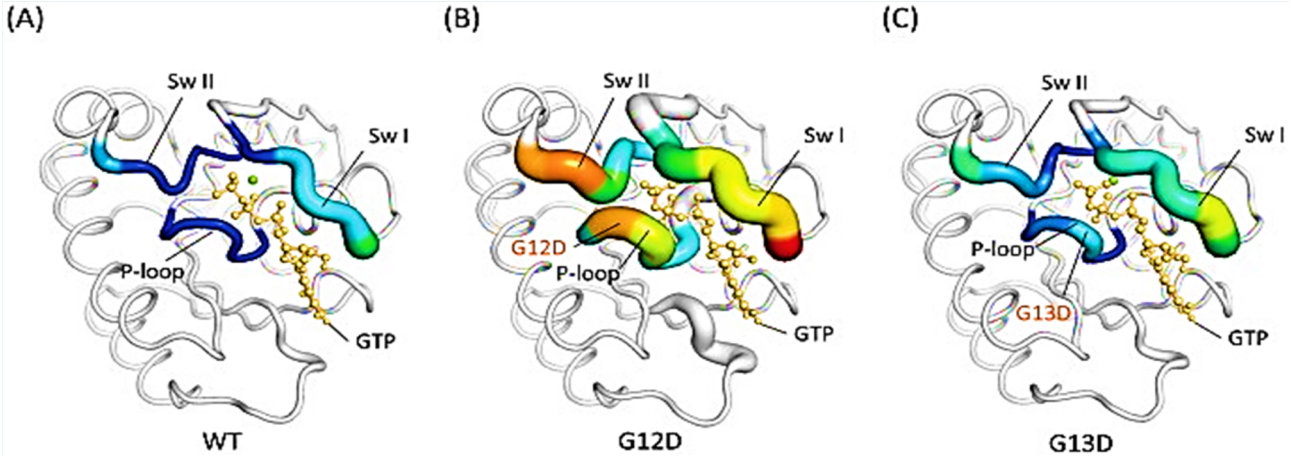

Fig. 4. The structures of (A) WT, (B) G12D and, (C) G13D KRAS proteins are drawn in cartoon putty representations at the P-loop, switch I and II regions; blue represents the lowest and red the highest B-factor value. In addition, the size of the tube reflects the value of the B-factor, in that the larger the B-factor, the thicker the tube. The structures in the other regions are coloured in white and displayed in cartoon tube representation, where the size of the tube is independent of the B-factors (Figure adapted from Chen *et al*., 2013).

inhibit each other (Aksamitiene *et al*., 2012). This cross talk between RAS-ERK-MAPK and PI13K is a context-dependent. Erk-dependent phosphorylation leads to post translational inactivation and disability of TSC2 to inhibit oncogenic progressions and mTOR signalling (Fig. 3). Activation of p38-kinase pathway and ERK are evident in lymphomas (Ma *et al*., 2005; Jazirehi et al., 2004; Kurland et al., 2003). Protein kinase C and RasGRP1/3 has been linked to apoptosis in B-NHL cells (Stang *et al*., 2009). In silico models as well as clinical trials have depicted that mutational status of K ras gene can affect the responsiveness to the currently available therapeutic agents for the anticancer treatment. By calculating the B-factors for each residue at P loop, switch I and II regions, Chen *et al*. (2013) observed that c.35G.A (p.G12D) has significant atomic fluctuations at the switch II and P-loop regions when compared with c.38G.A (p.G13D) and normal (Fig. 4).

Mutant Ras results in cellular instability and tumorigenesis through different mechansims (Jinesh *et al*., 2018). Understanding the mutational status and molecular genetics by using modern techniques like exome sequencing, before the start of any anticancer therapy is vital and rational combinations of therapeutic agents can be tried out.

ETHICAL APPROVAL

The study was approved by the Ethical Committee of School of Biological Sciences, Lahore and Advanced Board of Studies and Research, University of the Punjab, Lahore, Pakistan.

Conflict of Interest

All authors state that there is no conflict of interest.

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