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Research Article

Mutational analysis of Erythropoietin gene and its enhancer in anemic cancer patients

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Abstract: Erythropoietin (Epo) is a crucial hormone which binds to a cell surface receptor, triggers a set of downstream genes and plays a role in the feedback mechanism involved in correcting cellular hypoxic situation in anemic cancer patients. Mutational status of Epo gene and its enhancer were scrutinized to understand the association between genetic aspects and susceptibility of cancer patients to anemia. PCR followed by SSCP and sequencing was carried out to analyze the mutational status of 225 anemic cancer patients and 130 healthy controls. Odds ratio (OR), Chi square test (χ^2), 95% confidence intervals (CI) and relative risk (RR) were analyzed by Medcalc statistical software. Out of 225 cancer patients with anemia, 16 cases showed positive for acceptor splice site deletion mutation at intron2/exon3 junction and deletion of 'Adenine' at nucleotide 14 of exon 3 (at position 100,319,598) of Epo gene. Statistically, anemic patients carrying the mutation had 9.8 folds increased risk (OR-9.87; CI: 1.29- 75.35; *p*=0.02; RR-9.24). In Epo enhancer region, homozygous transition mutation (C/G to T/A) was observed 23bp upstream of hypoxia responsive element (HRE) in 31 patients, 2 controls and prevalence was highest in cervical cancers (70.96%). Anemic cancer patients carrying homozygous C/T mutation had 10.2 times increased risk of predisposition to higher anemic grades. (OR-10.22; CI: 2.40-43.47; *p*=0.001; $\chi 2$ =13.22; RR- 8.95). Though these mutations exert negative effect, their direct effect on hemoglobin and Epo levels seems to be minimal. We hypothesize from our findings that genetic predisposition can hasten individuals from developing anemia and that variations in the genetic status can predispose cancer patients to develop different grades of anemia.
Keywords: Erythropoietin, Mutations, Hypoxia, Cancer.

INTRODUCTION

Anemia is commonly observed in about 60-75% of the cancer patients. For most of them in advanced stages, treatment options are limited and mainly palliative [1]. Studies on erythropoietin (Epo) have helped in paving a way to understand the underlying mechanism involved in oxygen sensing mechanism of the body. Two decades of research on understanding the switch that regulates oxygen levels in response to hypoxia could elucidate the functional regions in charge of this control mechanism. Epo enhancer which is located 3' to the Epo gene was found to be responsible for hypoxia induced Epo gene regulation [2]. Central to the DNA binding transcription factor assembly on the enhancer is hypoxia inducible factor 1(HIF1), which harmonizes cellular oxygen levels during anemic conditions by upregulating the transcription of Epo gene [3-5]. This gene consists of 5 exons spanning a region of about 2.9kb DNA and translates into a 193 amino acids polypeptide which forms the Epo protein. This hormone is expressed by kidney and in part by the liver. The divergent expression pattern was studied in transgenic mice which revealed that the transcription initiation sites located around the Epo gene are differentially employed to express Epo mRNA [6,7]. Sequence located 6-14 kb upstream to the gene (5'-promoter) was found to be responsible for expression of the protein in kidney and immediate downstream sequence of the Epo gene (3'-flanking enhancer) played a role in Epo expression in the liver [8]. Out of the three motifs located in the enhancer region, a strong DNA-protein interaction was reported to form between the direct repeat of two steroid hormone receptor half sites separated by 2bp (DR-2 element) (binding site 5'-TGACCTCTCGACCC-3') and proteins released in response to normoxic or hypoxic conditions [9]. Whereas interaction of HIF-1 was shown at 3' end of minimal Epo enhancer region called the hypoxia responsive element (HRE) (binding site 5'-

TACGTGCT-3') [10, 2]. Mutations, whether nucleotide substitutions or deletions, in the second functional motif of the enhancer (CACA repeat region) that connects the HRE and DR2 motifs also interfere with hypoxia-induced expression [11]. Mutations that eliminate HIF-1 binding eliminate Epo induction and hence, HIF-1 transcriptional machinery and its recognition sequence in the Epo enhancer form the central features of cellular oxygen sensing mechanism [12]. Genetic alterations of key genes, either inherited or a result of evolved mutations could play a role in development of disease or conditions associated with the disease. As per this understanding our aim was to analyze the sequence specific changes of the Epo gene and its enhancer in anemic cancer patients and controls. This is to understand the relation between observed mutations and susceptibility of cancer patients to anemia.

MATERIALS AND METHODS

In this study, 225 anemic cancer patients and 130 healthy controls were enrolled. 103 cancer patients without anemia were also considered for Epo exon3 mutational analyses. There are varying degrees of severity of anemia, typically based on Hemoglobin (Hb) levels. The National Cancer Institute (NCI) anemia classifications were used to rate the severity of anemia in our study [13]. The enrollment of cancer patients and measures followed were in accordance with the standards laid out by the ethics committee of the Hospital. Further, patients' were informed about the study and consent as voluntary participants was obtained in this investigation. The study group was of South Indian origin and of a particular region i.e. Andhra Pradesh. At the time of enrollment along with their medical history, proforma was also recorded, the characteristics of which were age, gender, dietary habits, occupation, history of previous malignancies, type of cancer they were suffering from, treatment modality, treatment status and QoL parameters. All the data was subjected to statistical analysis. About 3ml of blood was collected by venipuncture from anemic cancer patients and healthy volunteers, which formed our control group. The genomic DNA was isolated by salting out method [14] and stored at -20^0c until further analysis. PCR followed by SSCP (Single Stranded Conformational Polymorphism) and sequencing was carried out to analyze the status of Epo gene sequence.

Amplification of Epo exons and its enhancer region:

The details of primer sequences used for amplification of Epo exons and its enhancer are shown in Table 1. Primers were designed specifically to amplify the complete exon regions along with a part of the flanking intronic regions. 10 picomoles of each primer were used in 25 µL final volume of standard PCR master mix (fermentas). UCSC genome browser was employed to locate conserved amino acids across different exons.

Table 1: Primer sequences used for amplification of erythropoietin exon regions and its enhancer.

Amplified regions	Oligo sequence	Size of the PCR Product
Epo Exon 1	E1 F: CGCACACATGCAGATAACAG E1 R: GCTAAATCCCCGCTCAAAC	354bp
Epo Exon 2	E2 F: CTCTCAGCCTGGCTATCTGTTC E2 R: GAATGTGCTGGGGAAGGGGT	196bp
Epo Exon 3	E3 F: CTTCAGGGACCCTTGACTCC E3 R: ATCAGGCTCGCAAATGAGAT	185bp
Epo Exon 4	E4 F: CCCAGAGTCCACTCCCTGTA E4 R: AGCAGAAGTGTCCGCTCCTA	226bp
Epo Exon 5	E5 F: CGACCTCCTGTTTTCTCCTTG E5 R: TGTTGGTGAGGGAGGTGGTG	225bp
Epo Enhancer	EE F: GTGGTGGGAACCATGAAGAC EE R: CCTCCCTCTCCTTGATGACA	399bp

Epo – Erythropoietin, PCR- Polymerase Chain Reaction.

For the amplification of exons 1,4,5 initial denaturation was carried out at 94°C for 3 min followed by 32 cycles of secondary denaturation at 94°C for 45 sec, annealing at 52°C for 40 sec and elongation at 72°C for 50 sec followed by final elongation at 72°C for 7 min.

Similarly the optimized PCR conditions for amplification of exon 2 and 3 were: initial denaturation- 94°C for 4mins; followed by 32 cycles of secondary denaturation- 94°C for 1min; annealing- 60°C for 1min; extension- 72°C for 50sec. Final extension was carried out for 5 mins at 72°C.

The same conditions as used for exon 1, 4 and 5 were used for amplifying Epo enhancer region except for annealing temperature which was 54^0c instead of 52^0c. (Bio-Rad thermo cycler)

Mutation analysis

15% polyacrylamide gel was used to analyze mutations. It was prepared with the following

components. Water(MilliQ)-6.520ml; Acrylamide (50%)-2.4ml; 10x TAE Buffer-1ml; APS(10%)-70µl and TEMED-10 µl. 5 µl of pcr product was denatured at 95^0c for 5mins along with 10 µl of denaturation buffer and then snap cooled. This was loaded onto the gel and ran at 120v for 2 hours. The gel was then silver stained to observe mobility shifts. Samples showing a clear shift were further analyzed by sequencing. ABI 3130 genetic analyzer was used to generate high quality sequence data. Further, Chromas Pro software was used to scrutinize chromatogram files.

Statistics

Patient characteristics were expressed as percentages. Odds ratios (OR), 95% confidence intervals (CI), Chi square (χ^2) and relative risk (RR) ratios were analyzed by Medcalc statistical software for Windows, version 7.4.1.0 (MedCalc Software, Mariakerke, Belgium). Two tailed *p* value ≤0.05 was considered significant in all the analysis conducted.

RESULTS

Characteristics of cases and controls:

A total of 355 individuals have been studied for Epo gene mutations of which, 225 were anemic cancer patients, and 130 were healthy population which formed our control group. The mean age was 45.5 years for cases and 35 years for controls. When patient characteristics were observed, highest number of cases recorded were between the age group 30-60 years (68.9%), followed by 16.9% of the patients < 30 years and 14.23% of the patients > 60 years. To observe the distribution of anemic conditions in men and women in the cancer population, random enrolment of cancer patients with anemia willing to participate in the study was permitted and hence, samples were not gender matched with controls in this study. Of the 225 cases enrolled, anemia was noticed in 72.89% women and 27.12% men. When patients were categorized based on severity of anemia, more female cases were observed in mild, moderate and severe anemia whereas no gender bias was observed in life threatening anemic cancer patients. 51.2% of the grade IV cases were women and 48.8% of the cases were men. While 80.43% women and 19.56% men represented grade III anemia, grade II anemia was prevalent in 76.27% women and 23.72% men, grade I anemia in and 77.21%women and 22.78% men. Hb levels ranged from 10.9 to 2.5 g/dl in cancer patients with anemia.

When these patients were characterized by type of cancer they were suffering from, 49.77% of the cases recorded were gynecological malignancies (Carcinoma of breast, cervix, endometrium, ovary, uterus, vagina) followed by 12% hematological malignancies (Leukemia, Lymphoma, multiple myeloma), 11.56% head and neck cases (buccal mucosa, hypopharynx, mouth, tongue, neck, thyroid, post cricoid region), 11.1% stomach cancers, 9.33% lung cancer cases and 6.22% other carcinomas. It was noticed that 59.12% of the cases were neither under chemo nor under radiotherapy at the time of admission.

Epo exon sequence variations:

Of all the Epo exons screened for sequence variations, consistent sequencing results were observed only in exon3 studies. Out of 225 cancer patients with anemia, 16 cases showed positive for splice site mutation at intron 2/exon 3 and homozygous deletion of 'Adenine' (A/-) at nucleotide 14 of exon 3 (at position 100319598) of Epo gene (Fig 1, 2 and 3). Hence in addition to these samples, 103 cases with cancer but without anemia were also studied to see the distribution of these mutations in this patient group. Of the 103 cancer patients without anemia, 13 cases were found to have the same mutations as observed in cancer patients with anemia.

It was observed from UCSC Genome browser that 18 out of 29 amino acids of Epo Exon3 were highly conserved across 5 mammalian species. Theoretically, of the 193 amino acids Epo protein, this deletion mutation in exon3 should result in substitution of glutamic acid with aspartic acid at 58^{th} amino acid and a stop codon at 62^{nd} amino acid.

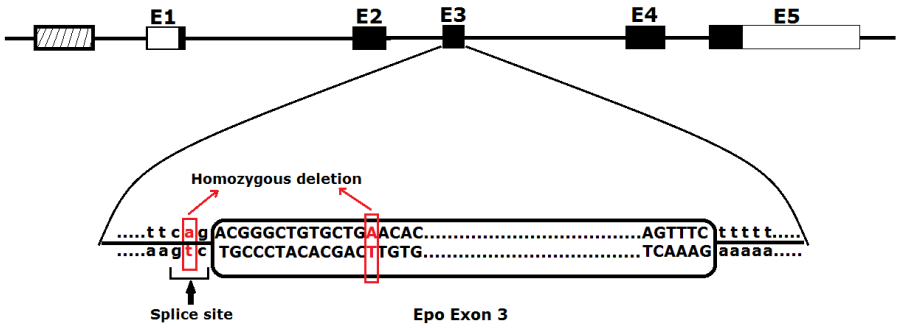

Figure 1: Mutations observed in erythropoietin exon3 region in anemic cancer patients.

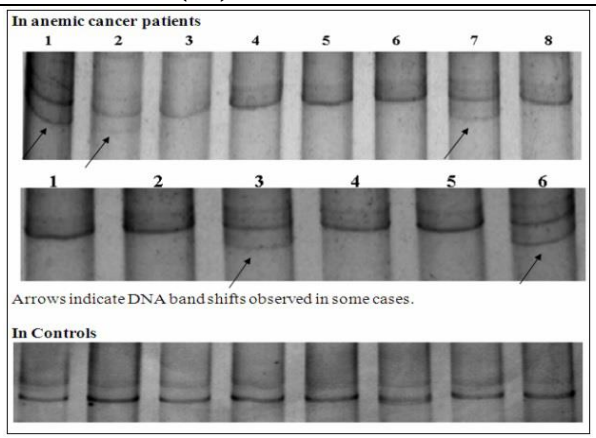

Figure 2: Single stranded conformational polymorphism studies of exon 3 of Erythropoietin gene. (Bands in lanes indicate denatured Exon3 PCR products, ran on polyacrylamide gels. Band shifts indicate variation/mutation in Exon3 sequence of Epo gene.)

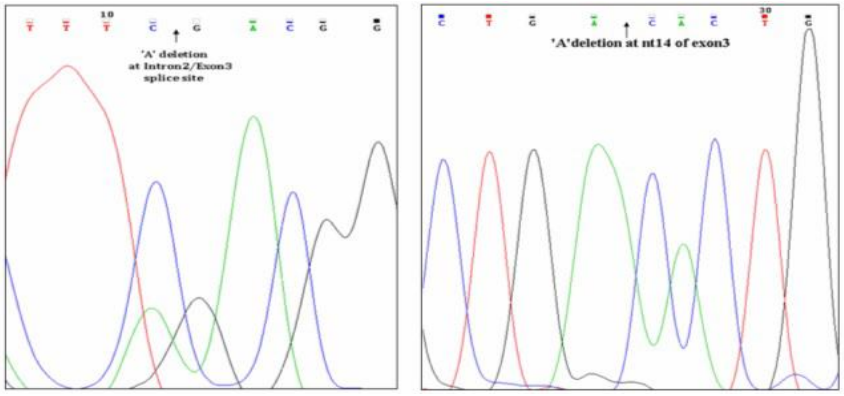

Figure 3: Chromatogram showing adenine deletion at intron2/exon3 acceptor splice site and in exon3 of Erythropoietin gene.

The prevalence of splice site mutation and A/- mutation was high in women (75%) compared to men (25%). Of the 16 cancer cases with anemia harboring the deletions at splice site and in exon 3, 9 cases were not under any treatment during sample collection, 4 cases were under chemotherapy and 3 cases were under radiotherapy. Hb levels ranged from 10.9 to 5.5 g/dl in these mutant cases. Similarly 56.25% of the mutant cases had grade I anemia whereas 18.75% each had grade II, grade III anemia and only 6.25% of the population suffered from grade IV anemia. 62.5% of the cases carrying mutations were between the age group 30-60 years. Deletion of 'Thymine' base at nucleotide10 of intron 3 was also observed but it was not statistically significant as it was seen in control population too.

Table 2: Genotype statistics of splice site mutation at intron2/exon3 junction and A/- on erythropoietin exon3.

Studied groups	**Acceptor splice site mutation at intron2/exon3 and deletion of 'A' at 14th nucleotide position of exon 3**		**OR**	**CI**	***P*-Value**	χ^2	**RR**
	Positive	Negative					
Control-Healthy population; n=130	1	129	0.10	0.13-0.77	0.02*	5.94	0.10
Cancer cases with anemia; n=225	16	209	9.87	1.29- 75.35	0.02*	5.94	**9.24**
Cancer cases without anemia; n=103	13	90	18.63	2.39 – 144.9	0.005*	12.27	**16.40**

n- Total number of cases; OR- Odds Ratio; CI- Class Interval; * p<0.05; RR- relative risk.

Anemic cancer patients carrying the mutation had 9.2 folds increased relative risk whereas cancer patients without anemia carrying the mutations were shown to have 16.4 folds increased risk of developing anemia (Table 2). This indicates that these mutations exert negative effect which is also evident from anemic

cancer patients' characteristics where 56.25% who were not under any treatment were still anemic at the time of sample collection.

Mutation analysis of Epo enhancer region:

Epo enhancer region which spans three motifs, important in the regulation of oxygen levels and in upregulating Epo transcription have been studied for sequence changes, the model of which is shown in fig 4. 225 cases and 130 controls were selected for the study. Homozygous C to T substitution was observed 23bp upstream of HRE in 31 patients and 2 controls. The SSCP and sequencing results depicting the homozygous transition mutation are shown in fig 5 and 6. The three conserved motifs of the enhancer region were free of any sequence changes in anemic cancer patients.

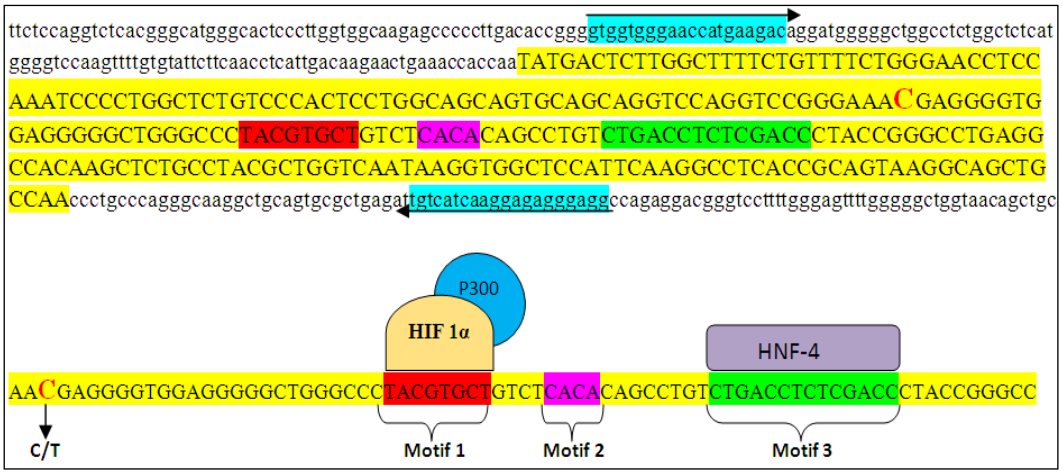

Figure 4: Details of Epo Enhancer region studied in anemic cancer patients and healthy individuals.

(Text highlighted in yellow depicts the enhancer region responsible for upregulating Epo gene. Text highlighted in red indicates hypoxia responsive element (HRE) which is critical for regulation of cellular oxygen levels by hypoxia. Second motif CACA repeat 7bp 3' to HRE highlighted in pink is important for proper function of enhancer. Third motif is marked by text highlighted in green which is direct repeat of two steroid hormone receptor half sites separated by 2bp (DR2) . Text highlighted in blue with arrows above and below indicates the primer binding region. Nucleotide 'C' marked in red color 23bp upstream of HRE was CG /TA mutation observed. Unhighlighted text marked in black indicates the sequence upstream and downstream of Epo gene.)

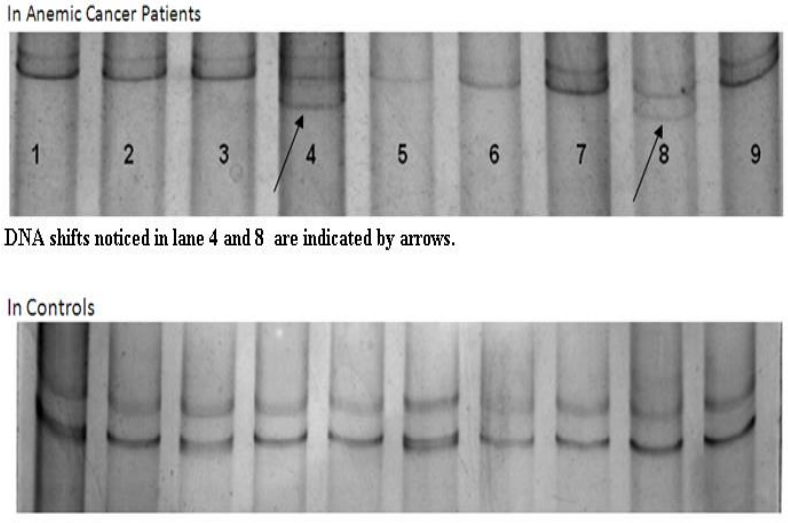

Figure 5: Mobility shifts observed on 15% polyacrylamide gel because of mutations in Epo enhancer region

(Bands in lanes indicate denatured Epo enhancer region PCR products, ran on polyacrylamide gels. Band shifts indicate variation/mutation in enhancer sequence of Epo gene.)

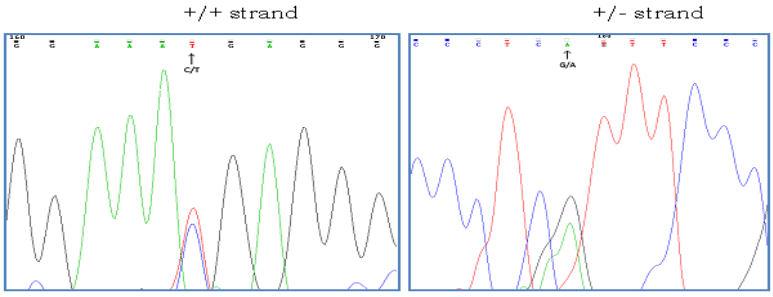

Figure 6: sequencing results showing *C/T* mutation in +/+ strand and +/- strand.

Anemic cancer patients carrying homozygous C/T mutation had 10.2 times increased risk of predisposition to higher anemic grades. (OR-10.22; CI: 2.40- 43.47; p=0.001; $\chi 2$ =13.22; RR- 8.95). The Hb levels varied from 10.9 to 7.2 g/dl in these mutant cases. Out of the 31 cases harboring the transition mutation, high prevalence was noticed in cervix cancer patients (70.96%) followed by 12.9% ovarian cases, 12.9% rectum and anus cases and 0.44% stomach cancers. 93.54% of the cases were between the age group 30-60 years and the rest were between 61-70 years of age. While 17 patients carrying the mutation in Epo enhancer region had mild anemia, 13 had moderate anemia and only 1 patient suffered from severe anemia. Life threatening or grade IV anemia was not noticed in any of the patients carrying the mutation.

DISCUSSION

It was clear from our studies that greater than 50% of the cancer patients were anemic even before the treatment which indicates that a subset of patients was more susceptible to anemia. We sought to analyze the genetic status of Epo gene and its enhancer as the susceptibility of cancer patients to anemic conditions can have its roots in this core gene which forms a vital part of the feedback mechanism involved in correcting cellular hypoxic conditions.

Of note, the prevalence of splice site mutation, exon3 mutation and C/T enhancer mutation was high in women compared to men (75% vs.25%). Out of all the patients harboring the C/T transition mutation, high prevalence was noticed only in gynecological malignancies, of note - cervix cancer patients (70.96%). This indicates that the genetic predisposition could be the cause for high incidence of anemia in women despite high iron levels.

Though women were predisposed to anemia as it was seen from the number of cases recorded, men suffering with cancer cannot be neglected as they appeared to be more susceptible to life threatening anemia. This was evident from our previous biochemical studies also where men were 7.6% more prone to grade IV anemia in cancers [15].

This splice site deletion mutation was observed at the invariant AG sequence of the splice acceptor site. This is the position at the 3' end of the intron where the intron is terminated in most eukaryotes. In such cases a nearby cryptic splice site may be used for termination of intron, post transcription, which might result in abnormally spliced mRNA transcript [16]. Mutations in splice sites can also result in an mRNA withholding large fragments of intronic DNA or entire exons being spliced out of the mRNA [17]. Though this single nucleotide deletion could lead to a truncated protein, this altered aberrant Epo protein seems to be still partially functional and is able to carry out functions through some other bypass mechanisms. To presume if splice variants play a role in maintaining the Epo levels, the splice variants of Epo have not been reported yet in humans. EpoR splice variants - soluble Epo receptor and EpoR-T have been first reported by Nakamura et.al [18]. These resulted from the intron being retained leading to an early stop codon or due to extended amino acid sequence. In the recent years other splice variants of EpoR have also been reported [19].

This study reports for the first time the role of splice site ‘A’ deletion mutation in anemic cancer patients. Further we also note that the levels of Epo were not influenced by this mutation as seen in our results.Surprisingly, existence of deletion mutation in the acceptor splice site of intron2/exon3 was observed in all the cases carrying adenine nucleotide deletion in exon 3. This indicates that these two mutations could be associated and playing a role together, hand in hand to generate a protein which would carry out the partial yet crucial functions.

Our study is also supported by the findings of Beck et.al [10], where they have shown hypoxia responsiveness even after deletion of DNA sequence from Epo exon1 to exon 5 and also 5’-flanking sequence which was highly conserved. This indicates that Epo enhancer was more crucial than the exon regions for its activity. In line with the observations, in our study, when the characteristics of the patients carrying the splice site and deletion mutation in exon3 region were observed, though the mutations have shown a significant result statistically, their direct effect on Hb levels or Epo levels seems to be minimal. Even the

transversion mutation noticed in the enhancer region has not shown any direct effect, as it is positioned away from the three vital motifs. Though these mutations did not have a direct effect, these patients carrying the mutations were susceptible to mild and moderate anemia.

This is the first case-control study analyzing Epo gene mutations in anemic cancer patients. A similar result with intron2/exon 3 acceptor splice site mutations, deletion of adenine nucleotide in exon 3, and CG to TA transversion mutation in enhancer, observed in our studies were not reported anywhere. Study of mutational analysis of important domains of Epo receptor and also enhancer binding regions of HIF gene along with this study would have had an added advantage in paving a way for drawing out conclusions to trace out the root cause of anemia in cancers.

CONCLUSION

It was evident from our studies that variations in the genetic status can predispose cancer patients to develop different grades of anemia. Why particularly women are more prone to anemia and why cervix cancer patients in particular are predisposed to Epo genetic alterations is yet to be explored. This study is an effort to find the connecting link between genetic aspects which are the master regulators of many diseases and anemia in cancers.

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