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IL-8 and IL-10 Gene Polymorphisms in North Indian Colorectal Cancer Patients: Association with mRNA and Plasma Levels - A Pilot Study

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ABSTRACT
Background: Colorectal cancer (CRC) is a significant global health concern. Still, the relationship between "IL-8-251 A/T & IL-10-1082 A/G polymorphisms" & CRC risk in North Indians is uncertain, & their effect on IL-8 & IL-10 levels is unknown. This investigation aimed to ascertain the prevalence of "IL-8-251 A/T & IL-10-1082 A/G polymorphisms" in North Indian CRC patients and their relationship to IL-8 & IL-10 mRNA & plasma levels.
Methods: The study investigated the relationship between "IL-8-251 A/T & IL-10-1082 A/G polymorphisms" & CRC risk in North Indian CRC patients. It examined mRNA & plasma levels of IL-8 & IL-10, compared them to healthy controls, & identified single nucleotide polymorphisms (SNPs) as potential risk factors for CRC. The study also investigated the mRNA expression patterns of IL-8 & IL-10.
Results: A study including 43 colorectal cancer patients & 65 controls discovered that tumor samples included more significant levels of IL-10 & IL-8 mRNA expression. The study also found a significant increase in IL-8 mRNA expression in CRC patients' tumor colonic mucosa. Genotyping of peripheral blood leucocytes identified IL-8 & IL-10 SNPs in both patients & healthy controls. Although IL-8 genotype & allele frequencies were not statistically different, IL-10 promoter polymorphisms were significantly greater in CRC patients.
Conclusion: The study found that colorectal cancer-infiltrating cells release inflammation-related cytokines, with pro-inflammatory cytokines increasing with tumor growth. Variant IL-10 alleles & genotypes are linked to CRC susceptibility risk, but there is no link with the IL-8-251A/T gene polymorphism in Indian patients.

Key-words: Colorectal cancer, Interleukin-8, Interleukin-10, mRNA, Plasma level, Single nucleotide polymorphisms

INTRODUCTION

CRC is a serious global health concern, with affluent countries having a greater incidence & fatality rate than poor countries [1]. However, new research indicates that the incidence of CRC is rising in India, making it a growing public health concern [2]. A complex combination of genetic & environmental variables influences the onset & progression of CRC. SNPs in genes producing cytokines have been identified as possible CRC risk factors.

"Interleukin-8 (IL-8)" is a pro-inflammatory cytokine that promotes angiogenesis, proliferation, & metastasis of CRC cells in the tumour microenvironment [3]. The IL-8 gene has multiple SNPs, one of which, the -251 A/T polymorphism, has been linked to a higher risk of CRC in diverse populations [4]. "Interleukin-10 (IL-10)" is an anti-inflammatory cytokine that reduces immune response & tumor cell proliferation [5]. The IL-10 gene also contains

many SNPs, including the -1082 A/G polymorphism, which has been linked to an elevated risk of CRC in several studies [6].

Genetic polymorphisms in the genes that encode IL-8 & IL-10 have been found & linked to an increased risk of developing CRC. The "IL-8-251 A/T polymorphism" (rs4073) is located in the promoter region of the IL-8 gene & has been demonstrated to impact IL-8 expression levels, with the T allele related with increased IL-8 production [7]. Similarly, the IL-10-1082 A/G polymorphism (rs1800871) is located in the IL-10 gene's promoter region & has been demonstrated to impact IL-10 expression levels, with the G allele related to decreased IL-10 production [8].

Several researchers have looked into the relationship between these polymorphisms & CRC risk, however, the results have been conflicting. Furthermore, there is minimal information on the relationship between these polymorphisms & "IL-8 & IL-10 mRNA" & plasma levels in CRC patients in North India. As a result, this study aims to explore the relationship between the "IL-8-251 A/T polymorphism" & the "IL-10-1082 A/G polymorphism" & mRNA & plasma levels of IL-8 & IL-10 in North Indian CRC patients. Understanding this relationship may provide light on the involvement of these cytokines in CRC etiology & help to create new therapeutic options for CRC care.

MATERIALS AND METHODS

The patients, who were scheduled to have surgery were enrolled in this study. Tumor tissue was collected from PGIMER's general surgery OT in Chandigarh for cytokine gene expression from thirty CRC patients who had undergone surgery. Unaffected colonic mucosa tissues from all patients were collected concurrently with tumor specimens to serve as normal control tissue. A 5 ml venous blood sample was collected from 43 CRC patients & 65 controls for IL-10 & IL8 promoter polymorphism isolation. Patient information, including smokers/non-smokers, alcohol intake, family history, & clinical history, was documented.

Inclusion criteria- The study included patients aged between 18 to 75 years & who had a confirmed diagnosis of colorectal cancer.

Exclusion criteria- Pregnant or breast-feeding women were excluded from the study.

Methodology

RNA preparation: Cellular RNA isolation- For RT-PCR applications, total cellular RNA was extracted according to the instructions provided by the manufacturer using TRI reagent (1 ml/10^7 cells) and a standard procedure conducted under RNase-free circumstances. RNase-free DNase treatment was used to eliminate contaminated genomic DNA. To reverse transcript, 1×reverse transcriptase buffer was mixed with 3 µg of RNA that had been reprecipitated using acid/salt/ethanol ("1 volume of RNA in aqueous solution, 0.08 volume of 3 M Na-acetate, 5.4 pH, 3.3 volume of 100% ethanol, -20°C for 16 h").

Reverse transcription & PCR amplification- Thermo Scientific's RevertAid First Strand cDNA Synthesis Kit was used for reverse transcription (RT) and PCR. Using 3 µg of cellular RNA as a template, cDNA was produced. We bought primers for the genes β-actin, IL-8, and IL-10. Primer-specific amplification of cytokines and β-actin cDNA (2 µl of 1/10 diluted stock) was performed. The sense (S) and antisense (AS) primer sequences, along with the amplicon lengths (L), are displayed in Table 1. The primers were made with cytokine cDNA as their specific target. A 2% agarose gel containing 10 µl of amplified DNA was electrophoresed with 0.5 µg/ml ethidium bromide in a Trisborate/EDTA solution. To see the bands, a UV transilluminator was utilized. The photos were analyzed with Bio-Rad's Molecular Analyst program.

DNA extraction & genotyping of IL-8 -251 A/T & IL-10 -1082 A/G SNPs- Genomic DNA was isolated from the peripheral blood leucocytes of 43 CRC patients & 65 healthy controls using standard Proteinase K digestion & the phenol-chloroform extraction procedure. "IL-8 -251 A/T & IL-10 -1082 A/G SNPs" were genotyped in both controls & patients using PCR-RFLP. SNP primers were constructed, & restriction enzymes were purchased (Table 2). PCR amplification was standardized using gradient PCR. The amplified products of IL-8 & IL-10 SNPs were digested with appropriate restriction enzymes, electrophoresed in 3% agarose gel, & stained with ethidium bromide.

Table 1: The annealing temperature, sequences of sense (S) & antisense (AS) primers & the lengths (L) of the amplicons of IL-10, IL-8, & β-actin

Gene Name	Primer Sequence	Annealing Temperature	Amplicon Length (bp)
IL-10	FP 5'- ATGCTTCGAGATCTCCGAGA -3' RP 5'- AAATCGATGACAGCGCCGTA -3	62.0^0C	269
IL-8	FP 5'- TTGGCAGCCTTCCTGATT -3' RP 5'- AACTTCTCCACAACCCTCTG-3	61.2^0C	247
β-actin	FP 5'- TCTACAATGAGCTGCGTG -3' RP 5'- CCTTAATGTCACGCACGA-3	60.0^0C	372

IL-8: Interleukin-8; IL-10: Interleukin-10; bp: Base pair

Table 2: Primer sequences & restriction enzymes used for genotyping IL-8 & IL-10 polymorphisms

Gene Name	Polymorphism	Primer Sequence	Restriction Enzyme
IL-8	-251 A/T	FP 5'- TCATCCATGATCTTGTTCTAA -3' RP 5'- GGAAAACGCTGTAGGTCAGA -3'	MfeI
IL-10	-1082 A/G	FP 5'-CCAAGACAACACTACTAAGGCTCCTTT-3' RP 5'-GCTTCTTATATGCTAGTCAGGTA -3'	XagI

IL-8: Interleukin-8; IL-10: Interleukin-10

Measurement of plasma IL-8 & IL-10 levels using enzyme-linked immunosorbent assay- Plasma was collected & centrifuged at 1500 rpm for 10 minutes. Plasma levels of IL-8 & IL-10 in controls & CRC patients were identified using commercial enzyme-linked immunosorbent assay (ELISA) kits designed particularly for these cytokines (R&D Systems, Inc., Abingdon, UK).

Statistical Analysis- Descriptive data were presented as mean±SD. In comparison, inferential statistics were determined through the Chi-square test in SPSS software and $p < 0.05$ were considered statistically significant.

Ethics approval and consent to participate- The institutional ethics committee authorized the study, and each patient provided informed permission.

RESULTS
There were 29 males & 14 females among 43 colorectal cancer patients, & 33 males & 32 females among 65 controls for "IL-8 -251 A/T & IL-10 -1082 A/G SNPs". Table 3 shows that the patient's mean age was 51.3±14.6 yrs & that of controls was 48.2±13.9 yrs.

Table 3: Age & sex distribution of CRC patients & controls

Sex	CRC patients (n=43)	Controls (n=65)
Males Numbers (Age Range)	29 (18-75 yrs)	33 (18-75 yrs)
Females Numbers (Age Range)	14 (32-70 yrs)	32 (40-66 yrs)

CRC: Colorectal cancer

The mRNA expression of IL-10 & IL-8 was measured in tumor & surrounding mucosal tissue from 30 CRC patients. Cytokine-specific mRNA levels were determined using RT-PCR in both tumor & surrounding colonic mucosa samples. Representative ethidium bromide-stained agarose gels displaying cytokine-specific DNA bands for IL-10 & IL-8 are depicted in Fig. 1A and B. More tumor samples contained pro- & anti-inflammatory cytokines, including IL-8 & IL-10 mRNA transcripts than the surrounding colonic mucosa did. However, IL-8 mRNA transcripts were overexpressed in most tumor tissues compared to the neighboring mucosa.
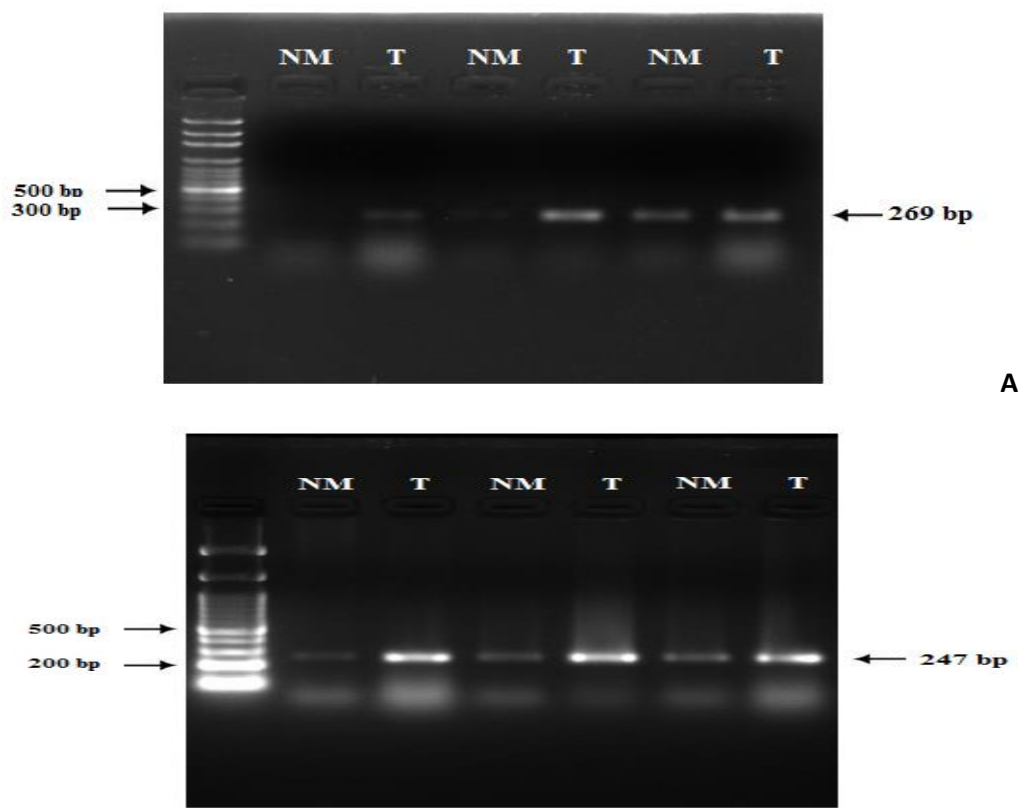
Fig. 1. Representative figures of (A) IL-10, & (B) IL-8 gene expression was analyzed in tumor tissue and normal mucosa obtained from patients with colorectal adenocarcinoma. Amplified DNAs were separated using a 2% agarose gel containing 0.5 µg/ml ethidium bromide.

IL-10 & IL-8 mRNA expression was detected in CRC patients' tumors & surrounding colonic mucosa. When compared to adjacent colonic mucosa, IL-8 mRNA expression increased the most, by 9.19-fold. Compared to neighboring colonic mucosa, IL-10 mRNA levels increased 1.97-fold (Table 4).

Table 4: Fold increase in cytokine mRNA expression in colorectal carcinoma tumor mucosa compared to adjacent mucosal tissue

Cytokine	Fold increase
IL-10	1.97
IL-8	9.19

IL-8: Interleukin-8; IL-10: Interleukin-10

Genotyping was done on isolated DNA from the peripheral blood leucocytes of 43 CRC & 65 healthy controls. The amplified products of IL-8 & IL-10 SNPs were digested with the appropriate restriction enzymes before electrophoresis in a 3% agarose gel stained with ethidium bromide (Fig. 2 & 3). For IL-8, PCR products were digested with 5 units of MfeI enzyme (MBI Fermentas) at 37^0C for 4 h, yielding 450 + 92 & 542 bp

digestion products for the A & T alleles, respectively. The visualization was done using 3% agarose gel electrophoresis. Lane M was loaded with 100 bp molecular marker. To digest the PCR results for IL-10, five units of the XagI enzyme (MBI Fermentas) were used for four hrs at 37^0C. The resulting digestion products for the A and G alleles were 280+97 & 253+27 bp, respectively. The visualization process was carried out using electrophoresis on 3% agarose gel. Lane M had a 100 bp molecular marker added.
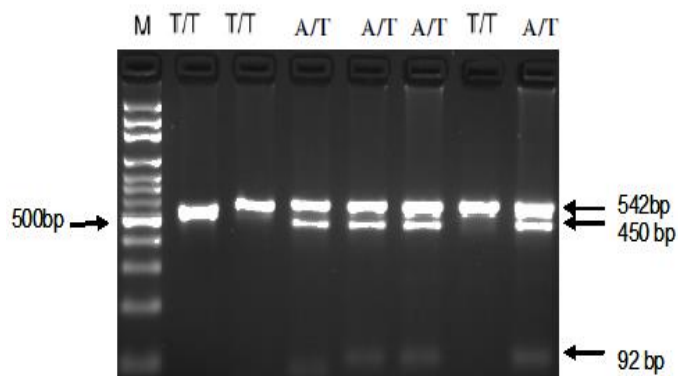

Fig. 2: Polymorphism analysis of IL-8 by PCR followed by MfeI restriction enzyme digestion.
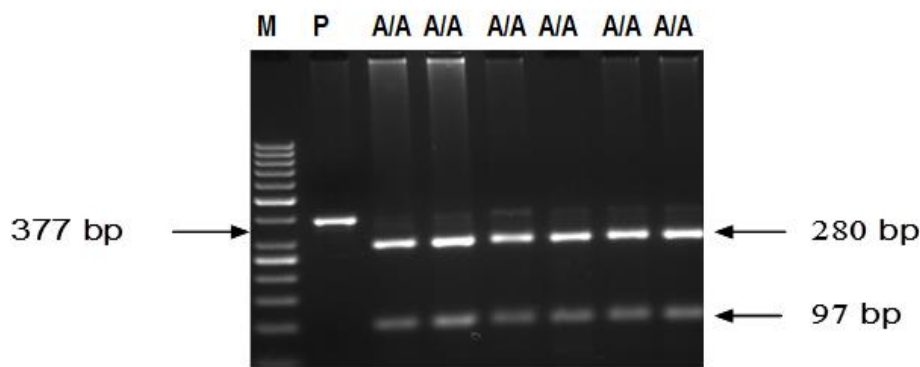
Fig. 3: Polymorphism analysis of IL-10 by PCR followed by XagI restriction enzyme digestion.

Further research revealed that the IL-8 genotype & allele frequencies of CRC patients were not significantly different from controls. For IL-8-251A/T, the genotype frequencies were AA 14%, TA 47%, and TT 40% (Tabel 5). On the other hand, CRC patients' IL-10 promoter polymorphism genotype and allele frequencies were substantially greater than controls (p<0.05). The genotype frequency of AA (83.7%) was lower than that of controls, but the genotype frequency of GA (16.3%) was higher in CRC patients. In CRC patients, the frequency of the mutant G allele (IL-10) was 91.8%, while in controls it was 99.2% (p<0.05). (Table 6). In this study, CRC patients' plasma levels of IL-8 and IL-10 were significantly higher than those of the healthy controls.

Table 5: Genotype & allele frequencies of IL- 8 promoter polymorphisms in colorectal cancer patients & healthy controls

Genotype	CRC (n=43) (%)	HC (n=65) (%)	p-value
IL-8-251			
TT	17 (40)	16 (25)	0.1515
TA	20 (46)	41 (62)	0.1332
AA	6 (14)	8 (13)	0.8032
A	32/86 (37.2)	57/130 (43.8)	NS
T	54/86 (62.8)	73/130 (56.1)	

If p<0.05 in chi-square test, it is accepted as statistically significant.

No statistically significant difference (IL-8-251 T>A polymorphisms) was determined between CRC & control groups (p>0.05).

IL-8: Interleukin-8; CRC: colorectal cancer; HC: healthy control.

Table 6: Genotype & allele frequencies of IL-10 promoter polymorphisms in colorectal cancer patients & healthy controls.

Genotype	CRC (n=43) (%)	HC (n=65) (%)	p-value
IL-10-1082			
GG	0 (0)	0 (0)	
GA	7 (16.3)	1 (1.5)	0.0128
AA	36 (83.7)	64 (98.5)	0.0128
A	79/86 (91.8)	129/130 (99.2)	
G	7//86 (8.2)	1/130 (0.76)	<0.05[a]

If p<0.05 in chi-square test, it is accepted as statistically significant.

[a]: Statistically significant difference (IL-10-1082 GA polymorphisms) was determined between CRC & control groups (p<0.05).

IL-10: Interleukin-10; CRC: colorectal cancer; HC: healthy control.

Table 7: Plasma levels of cytokines in colorectal cancer patients compared to healthy controls.

Cytokines	CRC patients (n=40)	HC (n=40)	p-value
IL-8 (pg/ml) **Mean ± SD**	30.6±13.25	14.7±0.95	0.04
IL-10 (pg/ml) **Mean ± SD**	7.3±3.02	4.4±0.69	0.01

IL-8: Interleukin-8; IL-10: Interleukin-10; CRC: Colorectal cancer; HC: Healthy control

DISCUSSION

The present study found that there were more male patients than female patients with colorectal cancer, which is consistent with other studies that have reported a higher incidence of colorectal cancer in men compared to women [9,10]. Previous studies have reported the association of IL-8 overexpression with CRC progression & poor prognosis [11,12]. Our findings support these studies, as we also observed IL-8 mRNA overexpression in most tumor samples compared to adjacent mucosa. This overexpression could potentially contribute to the inflammatory microenvironment & tumor growth in CRC. IL-10, an anti-inflammatory cytokine, has been shown to play a dual role in CRC, with both tumor-promoting & tumor-suppressive functions [13]. Our results demonstrate the presence of IL-10 mRNA transcripts in more tumor samples than in adjacent colonic mucosa. This observation is consistent with some studies suggesting that IL-10 might promote tumor growth & immune evasion in CRC [14,15]. However, other studies have reported that IL-10 can inhibit CRC progression by suppressing inflammation & Th1 responses [16,17].

The maximum fold increase of 9.19 in IL-8 mRNA expression in tumor tissue compared to adjacent colonic mucosa is consistent with previous studies. According to Harris *et al.* [18]*,* IL-8 is overexpressed in CRC & is associated with angiogenesis, tumor growth, & metastasis. Another survey by Kawada *et al.* reported that high levels of IL-8 are associated with poor prognosis in CRC patients [19]. The current findings support these previous studies & suggest that IL-8 may play a crucial role in the development & progression of CRC. The fold increase of 1.97 in IL-10 mRNA expression in tumor tissue compared to adjacent colonic mucosa is also consistent with previous studies, which reported that IL-10 is overexpressed in CRC & is associated with immune suppression, allowing tumor cells to evade the immune system [20].

Another study found that high levels of IL-10 are associated with poor prognosis in CRC patients [21]. The current findings support these previous studies & suggest that IL-10 may play a role in the immune suppression that allows CRC to progress.

For IL-8, the study identified two alleles (A & T) with digestion products of 450 + 92 bp & 542 bp, respectively. This is consistent with previous studies that have reported similar digestion patterns for IL-8 SNPs. For instance, a survey by Hull *et al.* [22] reported identical digestion patterns for IL-8 SNPs in investigating the association between IL-8 gene polymorphisms & colorectal cancer risk. In the case of IL-10, the study identified two alleles (A & G) with digestion products of 280+97 bp & 253+27 bp, respectively. This finding aligns with previous studies that have reported similar digestion patterns for IL-10 SNPs. For instance, a survey by Du Bois *et al.* investigated the association between IL-10 gene polymorphisms & colorectal cancer risk & reported similar digestion patterns for IL-10 SNPs [23].

The frequencies of IL-8 genotypes & alleles in CRC patients in the study were not statistically significant compared to controls. However, the genotype & allele frequencies of IL-10 promoter polymorphisms were statistically significant (p<0.05) in CRC patients compared to controls. This is consistent with other studies that have found an association between IL-10 promoter polymorphisms & an increased risk of CRC. A study by Hung *et al.* investigated the association between IL-8 & IL-10 gene polymorphisms & the risk of CRC in a Chinese population. The study found that the genotype frequency of IL-10 -1082A/G was significantly higher in CRC patients compared to controls (p<0.05), while the genotype frequency of IL-8 -251A/T was not statistically significant between the two groups [24]. Another study by Zhang *et al.* [25] investigated the association between IL-8 & IL-10 gene polymorphisms & the risk of CRC in a Chinese Han population. The study found that the genotype frequency of IL-10 -592C/A was significantly

higher in CRC patients compared to controls (p<0.05), while the genotype frequency of IL-8 -251A/T was not statistically significant between the two groups.

The study also found that plasma levels of IL-8 & IL-10 were significantly higher in CRC patients compared to normal controls, & the pattern of mRNA expression of IL-8, & IL-10 coincided with their respective plasma levels. This is consistent with other studies that have found an association between elevated plasma levels of IL-8 & IL-10 and an increased risk of CRC. A study by Wang *et al*. investigated the relationship between plasma levels of IL-8 & IL-10 & the risk of CRC in a Chinese population. The study found that plasma levels of IL-8 & IL-10 were significantly higher in CRC patients compared to normal controls (p<0.05) [26].

LIMITATIONS

The study did not identify the functional importance of "IL-8-251 A/T & IL-10-1082 A/G polymorphisms" about CRC risk. Also, it did not examine additional genetic or environmental factors that may impact colorectal cancer development & progression.

CONCLUSIONS

The current investigation reveals that CRC-infiltrating cells at the tumor site express inflammation-related cytokines. We investigated the cytokine gene expression pattern in patients with CRC. The local tumor microenvironment demonstrates a diverse expression of multiple cytokines, potentially forming a complex regulatory network. The current study also shows that gene expression of pro-inflammatory cytokines in the tumor microenvironment appears to be elevated as the tumor progresses. The present study found that mRNA expression levels correlated with plasma levels of pro- & anti-inflammatory cytokines. Both showed much higher amounts of cytokines than controls.

Furthermore, the present study found that the variant allele & genotype of IL-10 (G/A) were substantially linked with CRC susceptibility risk. However, there was a lack of connection between the "IL-8 -251A/T gene polymorphism" & CRC risk in Indian patients. Future study is needed to investigate additional genetic & environmental factors that might impact the development & progression of colorectal cancer in the North Indian population.

CONTRIBUTION OF AUTHORS

Research concept- Satyavati Rana
Research design- Rajesh Gupta
Supervision- Ram Rattan Negi
Materials- Satyavati Rana, Vikas Gupta, Rajesh Gupta
Data collection- Satyavati Rana
Data analysis and Interpretation-
Literature search- Vikas Gupta
Writing article- Vikas Gupta
Critical review- Ram Rattan Negi
Article editing- Satyavati Rana
Final approval- Ram Rattan Negi

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