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mtDNA Haplogroup M5 Associated with Risk of Colorectal Cancer in South India Population

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Abstract

The life on earth is driven by energy, supplied by the tiny organelles of the cell called mitochondria and they are usually stated as the powerhouses of the cell. In population genetics, Mitochondrial DNA (mtDNA) is used extensively to categorize individuals or populations. The mutation sites observed in human mtDNA by comparing with the reference sequence (rCRS) are termed into definite human mtDNA haplogroups. Previous studies showed that mtDNA specific haplogroups and polymorphisms were established to be linked with various human diseases, including cancer in numerous populations. Furthermore, it is also known that several mitochondrial DNA polymorphisms are implicated in enhanced production of Reactive Oxygen Species (ROS) which are known to be an increased risk cause for several cancers, including colorectal cancer in Indian patients. Hence in our study, we made high resolution examination on mtDNA hypervariable region to trace the association of specific mtDNA haplogroup with colorectal cancer in south Indian populations. We report that mtDNA Haplogroup M was observed in 60% of the colorectal cancer patients and around 55% in the studied control samples. Haplogroup M is the most frequent mtDNA cluster found among south Indian populations. We further sub-lineated macro haplogroup M and found sub haplogroups (M8, M7, M6, M5, M3 and M2) in varied frequencies. In particular, we found significant association of haplogroup M5 with colorectal cancer patients ($p = 0.026$). Haplogroup M5 was observed in 12% of colorectal cancer patients in south Indian patients and in 3.3% of the control populations. These results suggest that individuals with haplogroup M5 may have significant risk to develop colorectal cancer.

Keywords: Human mitochondrial DNA (mtDNA), Hypervariable region, Haplogroup M5, Colorectal cancer, South Indian population, D Loop

Introduction

Colorectal cancer (CRC) is described by uncontrolled cell growth in the appendix, rectum, or colon. The pathogenesis and etiology of colorectal cancer remains elusive despite numerous epidemiological studies categorized several factors for the cause of the disease [1, 2]. Globally, it is projected that CRC is the second highest prevalent cancer in females and third in males, with nearly 695,000 deaths and projection of over 14 lakhs (1.4 Million) of new cases each year till 2012 [3]. It is well established that genetic variations in nuclear DNA, with a specific emphasis on candidate genes, demonstrated strong association with colorectal cancer in various populations, including south Indian population [4]. It is to be noted that, like other cancers, CRC is also known to have strong multifactorial genetic basis [2]. With recent technological advancements in human exome sequencing, several studies pinpointed specific candidate genes [5, 6] connected with various cancers, including

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colorectal cancer. These studies also reported that APC (Adenomatous polyposis coli Gene) is the key candidate gene involved in colorectal cancer [5, 6]

Numerous studies explored the association of CRC with non-nuclear genome, i.e. the mitochondrial DNA. It was further proven that mutations in ROS encoding genes of human mitochondrial DNA may have increased risk in developing several cancers, including colorectal cancer. There has been a plethora of studies on nuclear DNA variations and colorectal cancer in humans, but there were only limited studies on the human mtDNA variations and colorectal cancer in south India. The unique set of mutations and polymorphisms observed in human mitochondrial DNA region, by comparing with Cambridge revised reference sequence (rCRS) in a group of individuals, is denoted as haplogroup [7,8]. Mitochondrial DNA is extremely unique in nature with no known recombination. It strictly follows maternal inheritance and undergoes high mutation rate and high copy number [9, 10]. The mtDNA has no repetitive DNA and does not contain introns or spacers. In total, human mtDNA hosts 37 coding genes which are all involved in the production and storage of energy molecules called ATP. The Human mitochondrial genome is 16536bp in length and encodes 2 rRNAs, 13 mRNAs, and 22 tRNAs. It is established that human mtDNA undergoes substitution rate that is higher than that of nuclear genome [11].

mtDNA haplogroup has an association with various neuromuscular diseases, cancer, diabetes and other diseases [12, 13, 14, 15, and 16]. However, human mtDNA haplogroup association with colorectal cancer in south Indian population is scarce. Mitochondrial DNA is called as the molecular clock. Within the structure of mtDNA there is a small non-coding region called the control region, which is known to carry the genetic signals needed for transcription and replication. By better understanding the control region of human mitochondrial DNA, we can estimate the relatedness between individuals depending on the base changes and DNA substitutions observed. The DNA sequence of the control region is termed hypervariable because it accumulates point mutations at approximately 10 times the rate of nuclear DNA. In the human control region, the estimate of the rate of substitution was found to range between 2.8 [17] to 5 times [18] the rate of the rest of the mtDNA. Most of the studies that used control region sequences focused on intraspecific patterns of variability and phylogenetic relationships of closely related species, a prominent example being the study of human population history [19]. Polymorphic nucleotide sites within this loop are concentrated in two hypervariable segments, namely the HVRI and HVRII [20]. Hence, HVSI and HVSII data can provide useful insights about inter- and intra-specific population variations. In the present study, we aim to understand the risk of mtDNA specific haplogroups with colorectal cancer in south Indian population by sequencing the two hypervariable regions (HVR1: np 16024–16383 and HVR2: np 57–333) of mitochondrial DNA in 100 cases and 90 control samples from south Indian population.

Materials and Methods

The sample collection for the present study to assess the mtDNA haplogroup association with colorectal cancer in south Indian population was performed from the Oncology department of Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India. 5ml of intravenous blood was collected in EDTA vacutainers from all the confirmed cases of colorectal cancer that also provided detailed informed consent. Age and gender data of the control and case samples are described in Table-3. Genomic DNA was isolated using phenol chloroform method as described earlier [21]. After the genomic DNA isolation from all the blood samples, the purity and integrity of the genomic DNA was tested using 1% agarose gel electrophoresis. Concentration of Genomic DNA was estimated by using UV Vis nanodrop spectrophotometer. Stock genomic DNA samples were stored in -20 and a working stock of 10ng/ul was made and stored for all the samples accordingly. Polymerase chain reaction was carried out using primers specific to human mtDNA hypervariable regions I and II. PCR products were electrophoresed in 2% agarose gel to check for the amplification by using specific 1KB ladder. PCR products were treated with EXO SAP and Big dye cycle Sequencing PCR reaction was then carried out as described by the manufacturer protocol. Both forward and reverse sequencing reactions were carried out separately in order to have the required read length. Post sequencing cleanup was carried out using ethanol precipitation and the products were denatured with formamide. Sequencing

products were then sequenced using Applied Biosystems 3730 DNA analyzer and bidirectional sequencing was carried out both for HVR I and II to obtain the complete required read length. The sequences were then base-called using Sequencing Analysis software. Mutations and polymorphisms were then analyzed using Auto Assembler software by the employment of Cambridge revised reference sequence (rCRS).

Statistical analysis

SPSS package (V11.0) was used to perform the required analysis. Fisher's Exact test was employed to test the Hardy–Weinberg equilibrium genotype distributions among the studied case and control samples. P value of 0.05 was applied as statistically significant. 95% confidence intervals were used for the calculation of odds ratio.

Phylogenetic analysis

Human mtDNA sequence of HVR I and II was used to build maximum parsimonious phylogenetic tree. Human mtDNA haplogroup was denoted to all the case and control samples in the present study based on the available literature [22, 23].

Results and Discussion

Colorectal cancer and mtDNA Control (D-loop) Region

The region in human mitochondrial DNA from base pairs of 16024 to 16576 (total length of 1121 bp) is called the control region, also known as the D- Loop region, which is the only non-coding part of entire mtDNA. It was stated that the non-coding region of human mtDNA is also called the hypervariable region because of the accumulation of point mutations at 10 times higher rate than that of the nuclear DNA. The high mutation rate is tolerated in the hypervariable region of mtDNA because the binding sites for DNA and RNA polymerase are distinct by only concise nucleotide sequences. It was also further stated that the observed high mutation rate in the hypervariable region of mtDNA may be due to the reason that mtDNA is situated in a neighboring vicinity to the respiratory machinery of the cell, which is called the oxygen free radicals. Therefore, the genetic variation observed in the hypervariable region may significantly affect the task of the respiratory chain which is accountable for enhanced levels of reactive oxygen species that could cause further nuclear genome destruction and cancer instigation and advancement [24]. Furthermore, it is evident that modifications in the respiratory chain may lead to mtDNA impairment in conditions like apoptosis induced by mitochondria [25]. Numerous studies examined the relationship of various cancers including the colorectal cancer with Somatic D- Loop mutations [24, 26, 27]. These reports indicated that carcinogenesis may occur due to instability of the human mtDNA D-loop, which is the non-coding portion.

In the present study, 179 variations were observed in the D-loop region with respect to the reference Cambridge sequence (Figure-1). Of these, 9 were novel mutations (Table-1) and 170 were reported mutations. Among the reported mutations, 5 were reported to be associated with various mitochondrial diseases. D-loop is the region which contains promoters, transcription factor binding sites, and other important regulating sites for translation and transcription of mt-DNA. Variations in this region were shown to be associated with several diseases. Overall, D-loop had 5.33 folds mutation rate (179 mutations/1121 NP =0.16) higher than the remaining part of mt-genome (527 mutations/15448 NP =0.03). Our results are consistent with most of previous reports which indicated that D-loop region has a mutation hot spot.

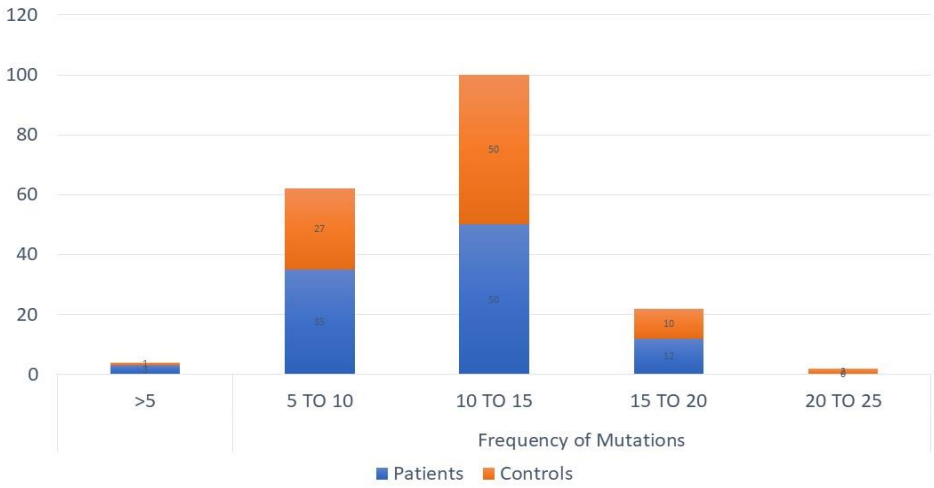
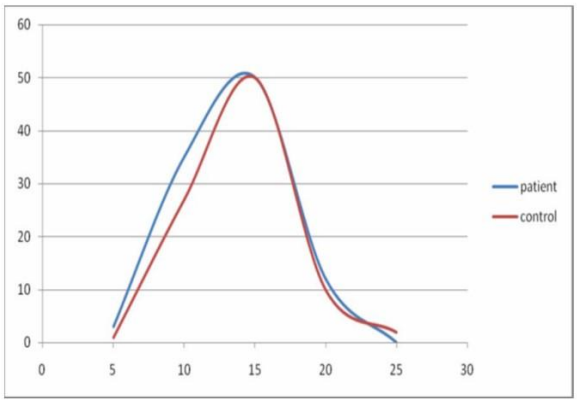


Figure 1: Distribution of mutations per individual in disease and control group of D-loop region

Reported mutations associated with other diseases

We also tested the variations observed in the HVR1 and HVRII regions of human mitochondrial DNA from the studied colorectal cancer patients to find out if the mutations observed have any association with other human diseases. Among the reported mutations, 5 were described to be related with various mitochondrial diseases (Table-2). Out of 9 novel variations, one patient had an insertion of cytosine at the 12th position and another patient had guanine insertion at position 16520. Eight patients had transition/transversion mutations. The variation C150T was found in association with longevity diseases, which was observed in 5 patients and 2 controls. The T16189C variant, described as susceptibility a factor in dilated cardiomyopathy, was detected in 14 patients and 13 controls. The transition mutation A189G, previously shown to be associated with leukemia, was observed in 3 patients and 3 controls. The T16519C mutation, associated with "cyclic vomiting syndrome with migraine", was observed in 76 patients and 65 controls.

In the D-loop region, the model class range was found to be 10-15 for both patients and controls (Figure-2), with an equal number of mutations (50). The area under the curves did not differ markedly between the patient and control groups (Figure-2). Furthermore, no association was observed with the clinical variables. The ratio of mutation frequency in the cases and control was found to be 1, which indicated that the accretion of mutations in the D-loop region alone may not confer risk to colorectal cancer (Figure-1).



MODEL CLASS FOR PATIENTS = 10-15
MODEL CLASS FOR CONTROLS =10-15

Figure 2: Distribution of D-LOOP region mutations among colorectal cancer patients and controls

As per the tumor/node/metastasis (TNM) classification of the American Joint Committee on Cancer (AJCC), the presently studied case samples are classified into stages 1, 2, 3 and 4. Distribution of mutations in HVR1 and HVR II of mtDNA in different stages in colorectal cancer patients are represented in Figure -3.

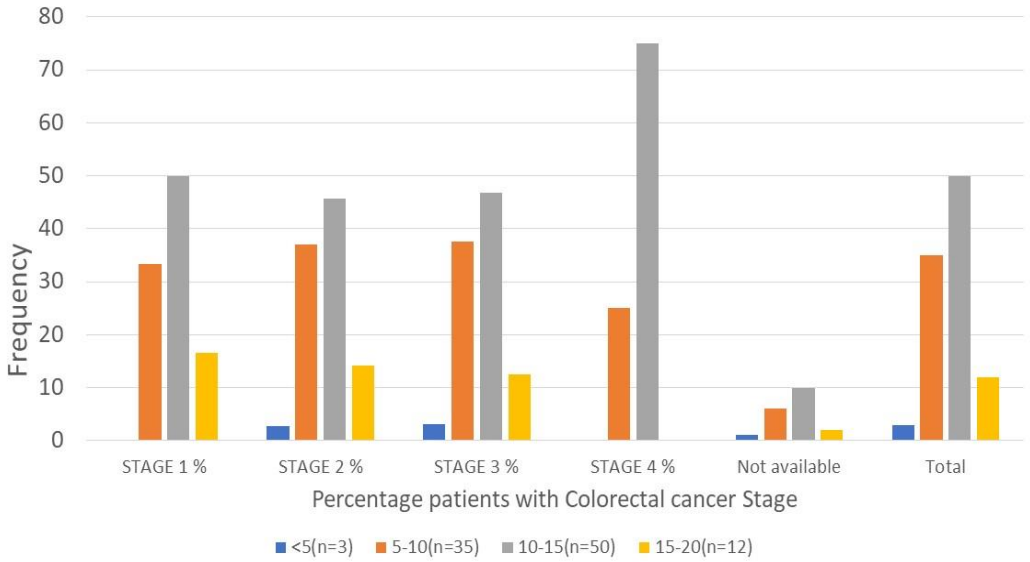


Figure 3: Distribution of Mutations with respect to stage of disease

Status of colorectal cancer with reference to macro haplogroups M and N

L, M and N are the major human mtDNA macro haplogroups, the distributions of which are geographically diverse. Out of the three major human mtDNA macro haplogroups, L is the oldest one and is primarily restricted to Africa continent, being more specially recorded in African Sub-Saharan Populations. Macro haplogroup L consists of the sub-haplogroups L7, L6, L5, L4, L3, L2, L1, and L0. Haplogroups M and N are the mtDNA haplogroups primarily observed in South Asian populations. It is widely established that around 60,000 YBP of L3 haplogroup is radiated out of Africa in the form of M and N macrohaplogroups, which paved the way to the present-day's South Asia [28,29]. Numerous population genetics studies using uniparentally transmitted genetic tools, such as Y chromosome and mtDNA, on Indian populations clearly elucidated that India played pivotal role in the early dispersal of human mankind. This is evident by the broad distribution of M and N haplogroups among many Indian populations [28, 29, 30, 31]. The distinct haplogroup of M consists of M50, M49, M48, M41, M40, M39, M38, M37, M36, M35, M34, M33, M32, M31, M30, M25, M18, M6, M5, M4, M3 and M2, which are Indian unique lineages. Indian populations represent both eastern and western Eurasian mtDNA haplogroups [31, 32]. The majority (~75%) of the Indian maternal pool consists of M and N, which spreads uniformly throughout India [28].

The absence of most of the Indian maternal mtDNA clades either in the adjacent Europe and East Asian populations or elsewhere in the world shows the autochthonous development of these haplogroups. It further indicates merely a restricted gene flow out from the Indian subcontinent over an extended period of time.

In our present case control study conducted on colorectal cancer patients in south Indian population, we investigated if there is any significant association between the major macro-haplogroup M and the incidence of colorectal cancer by sequencing the D -Loop region of mtDNA .In our results, mtDNA Haplogroup M was observed in 60 % of colorectal cancer patients and around 55% of control samples. Haplogroup M is the most frequent mtDNA cluster found among Indian populations [22, 23]. We further sub-lineated macro haplogroup M and found the sub haplogroups M8, M7, M6, M5, M4, M3, and M2 in varied frequencies (Figure -4, Table-4). In particular, we found a significant association of haplogroup M5 in colorectal cancer patients compared to the control samples ($p = 0.026$). Haplogroup M5 was observed in 12% of colorectal cancer patients and in 5% of the control population (Table-4).

India, being the corridor for early human dispersal across the world, has a population that is expected to have high levels of diversity in mitochondrial sequences. It was estimated that about 50 million individuals are affected with genetic diseases. However, only the genetic diseases of the nuclear origin were focused on so far, while there is no comprehensive study available on the mitochondrial diseases in India.

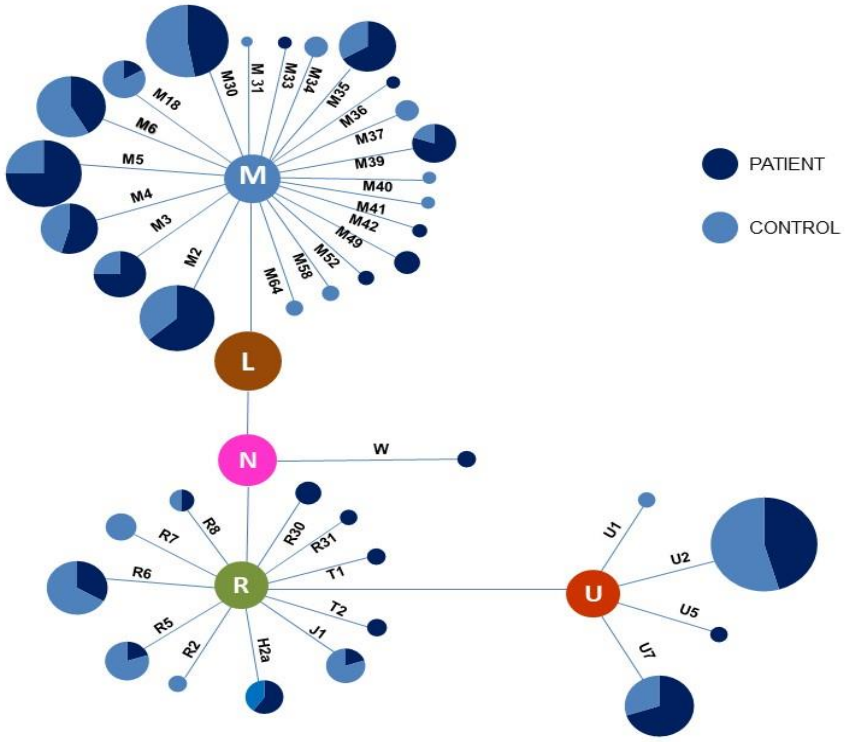


Figure 4-mtDNA haplogroup distribution in the studied patient and control samples.

Despite technological advancements and massive focus on oncology studies, there is still dearth of studies on understanding the molecular mechanism underlying the genetic causes of cancer and mtDNA polymorphisms. It was widely established that the major source of cellular energy synthesis and production, i.e. ATP, is driven by mitochondria, with any genetic alterations in mitochondrial DNA may have prominent impacts on the mitochondrial dysfunction.

Therefore, it is likely that mitochondrial deficiency could lead to nuclear genome instability as well. Furthermore, it needs to be defined that the importance of specific mtDNA polymorphisms need to be investigated with other nuclear polymorphisms in studies related to cancer epidemiology and the understanding of the genetic basis of cancer.

The present study identified that mtDNA haplogroup M5 is associated with the risk in colorectal cancer patients in south Indian population. As there is no recombination in human mitochondrial DNA, the detection of cancer by understanding the mtDNA mutations provides several benefits over that of nuclear DNA variations. It is known that mutations seen in human mtDNA are primarily homoplasmic and that the mitochondrial DNA content in tumor cells is higher compared to that in normal cells. Therefore, mtDNA mutations which are homosplasmic in nature characterizes a prevailing indication of clonality. The progress in better understanding the role of mtDNA polymorphisms in mitochondrial oncology' would be advantageous to obtain innovative markers which deliver valuable evidence in disease prevention. It would also be required to perform high resolution studies on the entire mtDNA genome sequencing to get deeper insights on haplogroup association with colorectal cancer in addition to the D Loop region of mtDNA. Therefore, it is noteworthy to further understand the role of mitochondrial mutations by sequencing the coding region of the entire human mtDNA for deeper insights on the mtDNA and colorectal cancer association.

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Author contributions: PVB and BPN conceived, designed, and wrote the manuscript. PVB analyzed the data and wrote the manuscript with inputs from BPN.

Conflict of interest: All the authors have read and approved the manuscript and there is no conflict of interest.

Table 1- Distribution of case and control samples according to the mtDNA haplogroup

HAPLOGROUP	CASE	CONTROL
H2a2a	3	2
J1	1	4
M	1	3
M18	1	5
M2	7	4
M3	6	2
M30	8	9
M31	0	1
M33	1	0
M34	0	2
M35	4	2
M36	1	0
M37	0	2
M39	4	1
M4	6	5
M40	0	1
M41	0	1
M42	1	0
M49	2	0
M5	12	4
M52	1	0
M58	0	1
M6	5	7
M64	0	1
R	7	2
R2	0	1
R7	0	3
R30	2	0
R31	1	0
R5	1	4
R6	3	6
R8	1	1
T1	1	0
T2	1	0
U1	0	1

U2	10	12
U5	1	0
U7	7	3
W	1	0
TOTAL	100	90

Table 2-mtDNA D- Loop mutations linked with other diseases

Gene	position of the nucleotide	Frequency	Disease association
D-Loop	C150T	5/2	Longevity/cervical carcinoma
D-Loop	A189G	3/3	leukemia
D-Loop	T16189C	14/13	type2 diabetes cardio myopathy
D-Loop	T16519C	76/65	cyclic vomiting syndrome with migraine

Table 3-Age and gender information of the studied case control samples

Age group and gender	Total patients N=100	Total controls N=90
>60 years	72	76
<60 years	28	14
Male	67	60
Female	33	30

Table 4- Human mtDNA haplogroups of case and control samples studied

Haplogroup	Number of patients	number of controls	%	χ^2	P-value
H2a	3	2	2.2	0.123	0.7258
J1	1	4	4.4	2.141	0.14341
M	1	3	3.3	1.23	0.26741
M18	1	5	5.5	3.115	0.07757
M2	7	4	4.4	0.593	0.44126
M3	6	2	2.2	1.761	0.1845
M30	8	9	10	0.222	0.63752
M31	0	1	1.1	1.1	0.29427
M33	1	0	0	1	0.31731
M34	0	2	2.2	2.2	0.13801

M35	4	2	2.2	0.523	0.46956
M36	1	0	0	1	0.31731
M37	0	2	2.2	2.2	0.13801
M39	4	1	1.1	1.649	0.1991
M4	6	5	5.5	0.022	0.88209
M40	0	1	1.1	1.1	0.29427
M41	0	1	1.1	1.1	0.29427
M42	1	0	0	1	0.31731
M49	2	0	0	2	0.1573
M5	**12**	**3**	**3.3**	**4.947**	**0.02614***
M52	1	0	0	1	0.31731
M58	0	1	1	1	0.29427
M6	5	7	7.8	0	0.43404
M64	0	1	1	1	0.29427
R 7	2	2	0.2	2	0.11356
R2	0	1	1.1	1	0.29427
R7	0	3	3.3	3	0.06928
R30	2	0	0	2	0.1573
R31	1	0	0	1	0.31731
R5	1	4	4.4	2	0.14341
R6	3	6	6.7	1	0.23489
R8	1	1	1.1	0	0.94363
T1	1	0	0 1	0	0.31731
T2	1	0	0 1	0	0.31731
U1	0	1	1.1	1	0.29427
U2	10	1	2 1	3	0.49437
U5	1	0	0 1	0	0.31731
U7	7	3	3.3	1	0.24898
W 1	0	0	1	0	0.31731
Total	100	90			

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