

RESEARCH ARTICLE

Novel Mutations in the Displacement Loop of Mitochondrial DNA are Associated with Acute Lymphoblastic Leukemia: A Genetic Sequencing Study

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is the most common cancer diagnosed in children and represents approximately 25% of cancer diagnoses among those younger than 15 years of age. **Materials and Methods:** This study investigated alterations in the displacement loop (d-loop) region of mitochondrial DNA (mtDNA) as a risk factor and diagnostic biomarker for early detection and diagnosis of acute lymphoblastic leukemia. Using mtDNA from 23 subjects diagnosed with acute lymphoblastic leukemia, the first 450 bp of the d-loop region were amplified and successfully sequenced. **Results:** This revealed 132 mutations at 25 positions in this region, with a mean of 6 alterations per subject. The d-loop alterations in mtDNA in subjects were all identified as single nucleotide polymorphisms in a homoplasmic distribution pattern. Mutant alleles were observed in all subjects with individual frequency rates of up to 95%. Thirteen mutant alleles in the d-loop region of mtDNA occurred with a high frequency. Novel alleles and locations were also identified in the d-loop of mtDNA as follows: 89 G insertions (40%), 95 G insertions (13%), 182 C/T substitutions (5%), 308 C insertions (19%), and 311 C insertions (80%). The findings of this study need to be replicated to be confirmed. **Conclusions:** Further investigation of the relationship between mutations in mitochondrial d-loop genes and incidence of acute lymphoblastic leukemia is recommended.

Keywords: Acute lymphoblastic leukemia - D-loop - mtDNA - mutations - genetic sequencing

Asian Pac J Cancer Prev, 15 (21), 9283-9289

Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer diagnosed in children and represents approximately 25% of cancer diagnoses among children younger than 15 years of age (National Cancer Institute, 2012 a,b). ALL occurs at an annual rate of 35 to 40 cases per 1 million people in the United States (Smith et al., 1999; National Cancer Institute, 2012a; 2012b). Among children and adolescents younger than 20 years of age, 2,900 are diagnosed with ALL each year in the United States (Smith et al., 1999; Dores et al., 2012). Over the past 25 years, there has been a gradual increase in the incidence of ALL (Shah and Coleman, 2007; National Cancer Institute, 2012a; 2012b).

A sharp peak in the occurrence of ALL is observed

among children of 2 to 3 years of age (more than 90 cases per 1 million per year), with rates decreasing to fewer than 30 cases per 1 million by the age of 8. The incidence of ALL among children of 2 to 3 years of age is approximately four times greater than that for infants and is four to five times greater than that for children of 10 years of age and older (National Cancer Institute, 2012a; 2012b). The incidence of ALL appears to be highest in Hispanic children (43 cases per 1 million), and the incidence is substantially higher in white children than in black children, with a nearly three times higher incidence from age 2 to 3 in white children than in black children (Smith et al., 1999; National Cancer Institute, 2012a; 2012b).

Awan et al. (2012) reported that the frequency of BCR-ABL FO in pediatric ALL, associated with poor overall

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survival. Their data indicated that an immediate need for incorporation of tyrosine kinase inhibitors in the treatment of BCR-ABL+ pediatric ALL in this population and the development of facilities for stem cell transplantation.

It is well known that mutational changes in the mitochondrial genome can be used as a diagnostic biomarker for early detection of cancer and as a potential target in the development of new therapeutic approaches. These findings strongly indicate that mtDNA mutations exert a crucial role in the pathogenic mechanisms of tumor development, but continued investigations are required to further elucidate the functional significance of specific mtDNA mutations in the etiology of human cancers (Yu, 2012).

Such studies using DNA divergence are becoming attractive in genetic population analysis, such as microsatellite loci, single nucleotide polymorphisms, and mitochondrial DNA (mtDNA) markers (Niu et al., 2002, Yacoub and Fathi, 2013).

Mitochondrial DNA is maternally inherited and does not undergo recombination; it is therefore a valuable molecule for investigating phylogenetic relationships among populations, subspecies, and species. It can also be used to evaluate the maternal genetic constitution for a specific population (Shen et al., 2002). The mitochondrial genome consists of 13 genes that encode 13 polypeptides (which are involved in oxidative phosphorylation), 22 tRNAs, and 2 rRNAs.

Mitochondria are important organelles that produce ATP through a vital pathway known as oxidative phosphorylation. This process is accomplished by a group of protein complexes and mitochondrial respiratory chains (MRCs), which are controlled by both nuclear and mitochondrial genomes (Higuchi, 2012). The mitochondrial genome of humans includes a high number of variant copies in each cell, and this number differs greatly based on the type of cell (Chatterjee et al., 2006). Mitochondria also serve fundamental functions in energy metabolism, production of reactive oxygen species (ROS), and apoptosis (Carew et al., 2003). Apoptosis itself is vital for cancer growth and in the cellular response to anticancer agents. The oxidative phosphorylation and cellular respiration pathways are also responsible for the generation of ROS, which are free radicals produced by oxygen metabolism. The close relationship between mitochondrial DNA and ROS generation makes mtDNA more sensitive to oxidative damage; this may lead to mutations in the mitochondrial genome and explain related cancer incidence (Chatterjee et al., 2006).

Various copies of the mitochondrial genome are found in each cell of humans and animals. Changes in mitochondrial DNA that are specific to only one of these genomes are known as heteroplasmic, to distinguish them from the normal cell type or the wild type. The percentage of heteroplasmy in the mitochondrial genome for each individual may also differ in time. Heteroplasmy can lead

to polymorphism between individuals within the same family, regardless of members carrying the same mtDNA mutation (Wallace, 1992; Carew et al., 2003).

Several studies demonstrate that mtDNA mutation is common in cancer (Lu et al., 2009; Cook and Higuchi, 2011). Mitochondria play vital functions in ATP metabolism, free radical generation, and regulation of apoptosis. Therefore, changes in mitochondrial DNA affect cellular energy capacities, increase oxidative stress, trigger ROS-mediated damage to DNA, and alter the cellular response to apoptosis induction by anticancer agents (Carew et al., 2003).

Many mutations in mitochondrial DNA have been characterized in different types of human cancers. Mutations in mitochondrial DNA have been seen in different regions within the same genome, and most of these mutations were reported as homoplasmic in nature (Chatterjee et al., 2006). In addition, mtDNA alterations activate mitochondria-to-nucleus retrograde signaling to modulate the expression of relevant nuclear genes or induce epigenetic changes that promote malignant phenotypes in cancer cells.

The aims of this investigation were to use alterations in the displacement loop (d-loop) region of mtDNA as a risk factor and diagnostic biomarker for early detection and diagnosis of acute lymphoblastic leukemia, and to determine the frequency of mtDNA variations in acute lymphoblastic leukemia in Saudi patients. This may make it possible to apply mitochondrial DNA mutations as a prognostic marker for the disease. This type of study has not been applied before on Saudi patients diagnosed with acute lymphoblastic leukemia, and is considered the first such report in the Kingdom of Saudi Arabia.

Materials and Methods

Subjects and acute lymphoblastic leukemia profile

Twenty-three subjects diagnosed between July 2009 and May 2013 with acute lymphoblastic leukemia (n=23; 16 males and 7 females) were involved in this study. Approval and consent were obtained from the Center of Excellence in Genomic Medicine Research (CEGMR) at King Abdulaziz University, in the Kingdom of Saudi Arabia.

Genomic DNA isolation

Genomic DNA samples were isolated from the subjects. Samples were deposited and stored in the biobank of the CEGMR at King Abdulaziz University.

D-loop Amplification of human mitochondrial DNA

Two primer sets designed by (Leiven et al., 1999) were used to amplify approximately 450 bp of the d loop region of human mitochondrial DNA, as listed in Table 1. PCR amplification reactions were performed on a 50 µl volume. This volume consisted of 50 ng of template

Table 1. Primer Pairs for Amplification of D-Loop Region of mtDNA

Pairs	Fragment name	Forward Primer Sequence	Reverse Primer Sequence
1	D-loop (Leiven et al., 1999)	CACCCTATTAACCACTCACG	TGAGATTAGTAGTATGGGAG

DNA, 10 pmol of each primer, 0.25 U of Taq DNA polymerase, 250µM of dNTPs mix, 10µM of Tris-HCl (pH 9.0), 30µM of KCl, 1.5µM of MgCl₂, and sterile nuclease-free water. PCR amplification was applied in a thermocycler (manufactured by Labnet International Inc). The following cycling conditions were used: pre-denaturation at 94°C for 5 minutes, denaturing at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles, and a final extension at 72°C for 10 minutes. The amplified fragments were analyzed by gel electrophoresis using a DNA ladder in order to assess the size of the amplificon product. The images were obtained using a gel documentation system (manufactured by Ultra-Violet Products Ltd.). The size of the amplicons was determined using software available with the gel documentation system.

Sequencing performance and sequencing analysis

The PCR products were purified and sequenced at Bioneer Inc. (Daejeon, Republic of Korea). The obtained sequences were aligned with GenBank (accession number NC_012920, GI 251831106) using nucleotide-nucleotide BLAST software and CLUSTALW version 2.0.12.

Homoplasmic and Heteroplasmic Identification

The homoplasmic and heteroplasmic variations were evaluated from obtained sequences of chromatograms.

Results

All PCR fragments of the d-loop region of mtDNA from subjects were successfully amplified, sequenced, and deposited in GenBank databases, as listed in Table 2. The results obtained from nucleotide-nucleotide BLAST software are shown in Table 2. A total of 23 subjects were studied. The subjects were from 2 to 43 years of age, with a mean of 14.2 years of age. The male to female ratio was 2:1.

A total of 132 mutations were found at 25 positions in the d-loop region, as shown in Table 2, with a mean of 6 alterations per subject. All variations in the d-loop region of mtDNA in the subjects were identified as single nucleotide polymorphisms (SNPs) with a homoplasmic distribution pattern, as noted in Table 2. No significant differences were found between male and female subjects in d-loop variation patterns and in SNP positions, but a higher number of d-loop alterations was observed for one female, who had 11 SNPs (BL-0938-12D, accession number KJ957906), as shown in Table 2.

Mutant allele frequencies in acute lymphoblastic leukemia

The normal and mutant allele frequencies of the d-loop region of mtDNA in subjects are shown in Table 3. Twenty-five variation sites in the d-loop region of mtDNA were recorded. The results show that the mutant alleles in the d-loop region of mtDNA were observed in all subjects, with individual mutation frequencies of up to 95%. Most of the mutant alleles had been previously identified in different reports, but some mutant alleles were previously undiscovered, as shown by a comparison with GenBank databases.

Table 2. Alterations in D-loop of Mitochondrial DNA in Acute Lymphoblastic Leukemia (ALL) Patients

Patient	SNP position	SNP No	Change	SNP type	Accession Number
BL-2366-10D	73	4	A/G	Homoplasmic	KJ957894
	263		A/G	Homoplasmic	
	309	C insertion	Homoplasmic		
	3011	C insertion	Homoplasmic		
BO-0744-11D	73	6	A/G	Homoplasmic	KJ957895
	152		T/C	Homoplasmic	
	207		G/A	Homoplasmic	
	263		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
	311		C insertion	Homoplasmic	
BL-2503-10D	73	5	A/G	Homoplasmic	KJ957896
	152		T/C	Homoplasmic	
	263		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
	311		C insertion	Homoplasmic	
BL-0147-10D	73	5	A/G	Homoplasmic	KJ957897
	204		T/C	Homoplasmic	
	207		G/A	Homoplasmic	
	263		A/G	Homoplasmic	
	311		C insertion	Homoplasmic	
BL-1055-10D	73	7	A/G	Homoplasmic	KJ957898
	150		C/T	Homoplasmic	
	195		T/C	Homoplasmic	
	263		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
	311		C insertion	Homoplasmic	
BI-0289-10D	263	4	A/G	Homoplasmic	KJ957899
	308		C insertion	Homoplasmic	
	309		C insertion	Homoplasmic	
	311		C insertion	Homoplasmic	
	316		G/A	Homoplasmic	
BL-2505-10D	73	9	A/G	Homoplasmic	KJ957900
	152		T/C	Homoplasmic	
	182		C/T	Homoplasmic	
	185		C/T	Homoplasmic	
	189		A/G	Homoplasmic	
	195		T/C	Homoplasmic	
	247		G/A	Homoplasmic	
	263		A/G	Homoplasmic	
	311		C insertion	Homoplasmic	
BL-0445-10D	73	6	A/G	Homoplasmic	KJ957901
	199		T/C	Homoplasmic	
	204		T/C	Homoplasmic	
	248		A deletion	Homoplasmic	
	263		A/G	Homoplasmic	
	311		C insertion	Homoplasmic	
BL-2290-10D	73	6	A/G	Homoplasmic	KJ957902
	146		T/C	Homoplasmic	
	200		A/G	Homoplasmic	
	263		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
	311		C insertion	Homoplasmic	
BO-0511-11D	73	5	A/G	Homoplasmic	KJ957903
	152		T/C	Homoplasmic	
	263		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
	311		C insertion	Homoplasmic	
BL-0849-12D	73	6	A/G	Homoplasmic	KJ957904
	89		G insertion	Homoplasmic	
	152		T/C	Homoplasmic	
	263		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
BO-0254-11D	73	6	A/G	Homoplasmic	KJ957905
	152		T/C	Homoplasmic	
	248		A deletion	Homoplasmic	
	263		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
	311		C insertion	Homoplasmic	

Table 2 (Cont). Alterations in D-loop of Mitochondrial DNA in ALL Patients

Patient	SNP position	SNP No	Change	SNP type	Accession Number
BL-0938-12D	73	11	A/G	Homoplasmic	KJ957906
	89		G insertion	Homoplasmic	
	189		A/G	Homoplasmic	
	195		T/C	Homoplasmic	
	204		T/C	Homoplasmic	
	207		G/A	Homoplasmic	
	210		A/G	Homoplasmic	
	263		A/G	Homoplasmic	
	308		C insertion	Homoplasmic	
	309		C insertion	Homoplasmic	
	311		C insertion	Homoplasmic	
BO-0845-12D	73	6	A/G	Homoplasmic	KJ957907
	89		G insertion	Homoplasmic	
	95		G insertion	Homoplasmic	
	263		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
BL-1093-12D	73	6	A/G	Homoplasmic	KJ957908
	89		G insertion	Homoplasmic	
	95		G insertion	Homoplasmic	
	195		T/C	Homoplasmic	
	263		A/G	Homoplasmic	
BL-2354-10D	89	6	G insertion	Homoplasmic	KJ957909
	146		T/C	Homoplasmic	
	263		A/G	Homoplasmic	
	308		C insertion	Homoplasmic	
	309		C insertion	Homoplasmic	
BO-0578-12D	73	8	A/G	Homoplasmic	KJ957910
	89		G insertion	Homoplasmic	
	95		G insertion	Homoplasmic	
	204		T/G	Homoplasmic	
	206		T/G	Homoplasmic	
BL-2506-10D	73	6	A/G	Homoplasmic	KJ957911
	89		G insertion	Homoplasmic	
	248		A deletion	Homoplasmic	
	263		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
Bo-2562-10D	73	5	A/G	Homoplasmic	KJ957912
	89		G insertion	Homoplasmic	
	246		A deletion	Homoplasmic	
	263		A/G	Homoplasmic	
	311		C insertion	Homoplasmic	
BL-0997-12D	73	5	A/G	Homoplasmic	KJ957913
	89		G insertion	Homoplasmic	
	200		A/G	Homoplasmic	
	309		C insertion	Homoplasmic	
	311		C insertion	Homoplasmic	
BL-0607-13D	146	6	T/C	Homoplasmic	KJ957914
	152		T/C	Homoplasmic	
	189		A/G	Homoplasmic	
	214		A/G	Homoplasmic	
	263		A/G	Homoplasmic	
BL-2328-10D	146	4	T/C	Homoplasmic	KJ957915
	263		A/G	Homoplasmic	
	308		C insertion	Homoplasmic	
	309		C insertion	Homoplasmic	

Novel mutations in acute lymphoblastic leukemia

Novel mutation sites and alleles were identified in the d-loop of mtDNA in this investigation as follows: 89 G insertions (40%), 95 G insertions (13%), 182 C/T substitutions (5%), 308 C insertions (19%), and 311 C

Table 3. Allele Frequency in Acute Lymphoblastic Leukemia (ALL) patients

SNP Position	Normal Allele	Mutant Allele
73 A/G	A	0.19
152 T/C	T	0.69
150 C/T	C	0.96
263 A/G	A	0.05
204 T/C	T	0.81
207 G/A	G	0.87
195 T/C	T	0.81
182 C/T	C	0.95
186 C/T	C	0.95
188 A/G	A	0.86
247 G/A	G	0.95
146 T/C	T	0.81
199 T/C	T	0.95
200 A/G	A	0.95
206 T/G	T	0.95
210 A/G	A	0.95
214 A/G	A	0.95
89 G insertion	()	0.60
95 G insertion	()	0.87
248 A/deletion	A	0.81
308 C insertion	()	0.81
309 C insertion	()	0.27
310 T/C	T	0.95
311 C insertion	()	0.20
316 G/A	G	0.95

Table 4. The Highly Frequent Mutant Alleles in Acute Lymphoblastic Leukemia (ALL) Patients

Mutation position	Mutant allele	allele frequency
263 A/G	G	0.95
73 A/G	G	0.81
311 C insertion	C insertion	0.8
309 C insertion	C insertion	0.72
89 G insertion	G insertion	0.4
152 T/C	C	0.31
146 T/C	C	0.19
308 C insertion	C insertion	0.19
204 T/C	C	0.18
195 T/C	C	0.18
248 A deletion	Deletion	0.18
95 G insertion	G insertion	0.13
189 A/G	G	0.13

insertions (80%), as listed in Table 3.

The most frequent mutant alleles in acute lymphoblastic leukemia

Thirteen mutant alleles in the d-loop region of mtDNA were observed with high frequency rates, as reported in Table 4. The most common mutant alleles were 263 A/G, 73 A/G, 311 C insertion, and 309 C insertion, which occurred at rates of 95%, 81%, 80%, and 72%, respectively. A moderate frequency was recorded for the novel mutation 89 G insertion (40%) and for 152 T/C (31%). The rest of the mutant alleles had low frequencies ranging from 13% to 19%.

Discussion

Many studies report that alterations in mitochondrial DNA play a fundamental role in diseases such as Leber's hereditary optic neuropathy, maternally inherited diabetes mellitus, and Leigh's syndrome (DiMauro and Schon, 2001). While these diseases are due to germline mutations, somatic mutations have been observed in other diseases,

especially cancer. The accumulation of somatic mutations is greater in mtDNA than in nuclear DNA because DNA replicates only at the time of cell division and undergoes proofreading by DNA polymerase. However, turnover of mtDNA is high, as degradation and replication is a continuous process in mitochondria, even within a single cell cycle, and mtDNA polymerase γ does not have the ability to proofread (Shadel and Clayton, 1997). The most relevant difference between the mitochondrial and nuclear genomes is therefore their inherent susceptibility to damage (Carew and Huang, 2002). In fact, it is well established that mtDNA is much more susceptible to alterations than nuclear DNA, due to its lack of histone protection, limited repair capacity, and close proximity to the electron transport chain, which constantly generates superoxide radicals that also cause genetic damage. Since mtDNA lacks introns, most mutations also occur in coding sequences and are thus likely to be of biological consequence (Zastawny et al., 1998). However, even mutations in the non-coding region of mtDNA may be associated with cancer incidence, as shown by alterations to the d-loop region, which regulates the replication and transcription of mtDNA (Sharawat et al., 2010).

Variations in the mitochondrial d-loop region co-occur with different solid malignancies, with a mutation frequency of 20% to 80% depending on the cancer type (Alonso et al., 1997; Ivanova et al., 1998; Nomoto et al., 2002; Tan et al., 2002; 2006; He et al., 2003; Suzuki et al., 2003; Grist et al., 2004; Lievre et al., 2005; Guo and Guo, 2006; Yao et al., 2007; Wulfert et al., 2008; Yun et al., 2009; Sharawat et al., 2010). Several studies have reported alterations in mtDNA in hematological malignancies. The first hematological mtDNA abnormalities were discovered by using cesium chloride-ethidium density centrifugation and electron microscopy to examine the structure of mtDNA in leukemic leukocytes (Clayton et al., 1967). The authors identified alternate mtDNA structures comprised of circular dimers, catenated dimers, and catenated trimers. While these alternate mtDNA structures can also exist in normal cells, the authors found an unusually high percentage of them in leukemic cells as compared to normal controls (Clayton and Vinograd, 1967).

In another investigation, the authors examined the leukocytes of 14 subjects with acute and chronic granulocytic leukemia. Circular dimers were found in all 14 subjects, but not in the leukocytes of 3 healthy donors.

It was demonstrated that the percentage of circular dimers decreased in some leukemic subjects following chemotherapeutic treatment, suggesting that the severity of leukemia may be related to the presence of circular dimers (Clayton and Vinograd, 1969). Analysis of mtDNA from acute myelogenous leukemia cells revealed that the origins of abnormal mtDNA structures could be traced back to the bone marrow (Robberson et al., 1981). Another report on leukemia cells from subjects with acute lymphoblastic leukemia identified mtDNA point mutations in 11 of 30 subjects (Ivanova et al., 1998). In this investigation, we used direct DNA sequencing on the d-loop region of mtDNA. This has an advantage over alternative techniques of identification, such as PCR-RFLP, in that PCR amplification using specific primers

is less expensive and more useful for routine analysis of large numbers of samples.

Shaikh et al. (2014) concluded that the relative lack of good prognostic cytogenetic abnormalities like t(12;21) (p13;q22) and hyperdiploidy (47-57 chromosomes) in Pakistani children with ALL. Prevalence of poor prognostic cytogenetic aberrations like t(9;22)(q34;q11.2) is comparable to available international literature.

Saber et al. (2012) reported that the frequency of 5 fusion oncogenes in adult ALL patients, and their association with clinical features, treatment response and outcome. Frequencies of some of the oncogenes were different from those reported elsewhere and they appear to be associated with distinct clinical characteristics and treatment outcome.

Soheila et al. (2013) indicated that the decrease in the type I error rate and increase the power in multivariate (Hotelling's T²) test as increasing the correlation between gene pairs in contrast to the univariate (Category) test.

Both homoplasmic and heteroplasmic mtDNA mutations have been observed in cancer cells. In our investigation, all mutations in the d-loop region of mtDNA in subjects with ALL were identified as single nucleotide polymorphisms with a homoplasmic distribution, which is the most common pattern of mtDNA mutations (Chatterjee et al., 2006). The mechanisms by which such homoplasmy arises from heteroplasmic mutations in cancer cells still remain to be defined. Coller et al (2001) used extensive computer modeling to suggest that if an mtDNA mutation occurs in a tumor progenitor cell, homoplasmy can be achieved entirely by chance through unbiased mtDNA replication and sorting during cell division, without selection for physiological advantage.

In conclusion, five mutation sites were identified in the d-loop region of mtDNA in subjects with acute lymphoblastic leukemia, with differing mutation frequencies (Table 3). These types of mutations have not been studied before. We compared these new mutations sites (89 G insertion (40%), 95 G insertion (13%), 182 C/T (5%), 308 C insertion (19%), and 311 C insertion (80%)) with the known and available databases of GenBank for mitochondrial d-loop region sequences. The comparison showed that these types of mutation sites have not been reported before, and they can be considered as novel mutations in the mitochondrial d-loop region. These mutations are associated with acute lymphoblastic leukemia, and therefore may be useful in diagnosis as risk factors for the disease. This investigation could also be used to provide an overview of the incidence frequency of ALL in Saudi patients.

Acknowledgements

We gratefully thank the Center of Excellence in Genomic Medicine Research (CEGMR) at King Abdulaziz University, Kingdom of Saudi Arabia, for providing us with DNA samples of subjects with ALL.

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