Analysis of single nucleotide polymorphisms in the FAS and CTLA-4 genes of peripheral T-cell lymphomas

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Abstract Angioimmunoblastic T-cell lymphoma (AILT) represents a subset of T-cell lymphomas but resembles an autoimmune disease in many of its clinical aspects. Despite the phenotype of effector T-cells and high expression of FAS and CTLA-4 receptor molecules, tumor cells fail to undergo apoptosis. We investigated single nucleotide polymorphisms (SNPs) of the FAS and CTLA-4 genes in 94 peripheral T-cell lymphomas. Although allelic frequencies of some FAS SNPs were enriched in AILT cases, none of these occurred at a different frequency compared to healthy individuals. Therefore, SNPs in these genes are not associated with the apoptotic defect and autoimmune phenomena in AILT.

Keywords Angioimmunoblastic T-cell lymphoma · Autoimmune diseases · Apoptosis · Single nucleotide polymorphism · FAS · CTLA-4

Introduction

Angioimmunoblastic T-cell lymphoma (AILT) is characterized by a systemic lymphoproliferative disorder, generalized lymphadenopathy, and immunologic abnormalities. Originally, it was debated whether the disease should be considered a lymphoma or, alternatively, an abnormal hyperimmune reaction because the fatal outcome was mostly attributed to severe infectious complications rather than to the lymphoproliferation itself. Besides a dispersed cellular infiltrate of atypical lymphoid cells, predominant histological features of AILT include an inflammatory background of plasma cells and eosinophils as well as a proliferation of “arborizing” vessels and follicular dendritic cells. AILT patients frequently exhibit autoimmune phenomena such as cold agglutinins with hemolytic anemia, circulating immune complexes, anti-smooth muscle antibodies, and positive rheumatoid factor [1].

In recent years, clonality analysis in AILT has demonstrated a clonal expansion of T-cells in most cases. Furthermore, studies focused on the immunophenotype of the tumor cells including their correlation with normal T-cell counterparts [2], underlying cytogenetic alterations, and associations with Epstein–Barr virus (EBV) infections and EBV-driven accom-
panying B-cell proliferations. Based on the expression of CXCL13 and CD10, tumor cells in AILT were correlated to germinal center B-helper T-cells, which represent a population of follicular T-cells constituting a crucial checkpoint for B-cell differentiation and for the maintenance of B-cell tolerance in the periphery [3–5]. However, few approaches have been taken to explain the autoimmune features that are prominent in many AILT patients.

We could recently demonstrate that the neoplastic T-cells in AILT homogeneously correspond to effector cells [2] who are by their natural fate destined to die from apoptosis. This is in contrast to peripheral T-cell lymphomas, not otherwise specified (PTCL-NOS) which correspond to central memory T-cells, a long-living T-cell population that develops by “below threshold” antigen stimulation or when polarizing cytokines are missing [6]. The programmed cell death in effector cells is indispensable for the termination of physiological immune reactions [7], and, interestingly, the tumor cells in AILT express the two molecules FAS and CTLA-4 which are mainly regarded as mediators of apoptosis induction in lymphocytes [8]. Furthermore, the neoplastic T-cells in most of the AILTs express CD10 [9], which is a neutral peptidase expressed in reactive T-cells and some neoplastic B-cells after induction of apoptosis [10–12]. BCL-2, an anti-apoptotic molecule, has been shown to be overexpressed in different B-cell lymphomas [13], while it is consistently negative in AILT [2]. Taken together, these data raise the question, why the neoplastic T-cells in AILT do not undergo apoptosis.

In mouse models, pathological features resembling those in AILT can be induced by introducing mutations of the FAS [14], the CTLA-4 [15], or effector-caspase genes [16], respectively. Moreover, the morphological changes in lymph nodes of patients with an autoimmune lymphoproliferative syndrome (ALPS), a disease caused by hereditary mutations in the FAS gene [17, 18], can mimic the histology of AILT.

FAS, a homotrimeric transmembrane receptor, is an important mediator for the downregulation of immune responses [19] by inducing apoptosis of antigen-primed lymphocytes, including those with autoimmune potential [20]. The gene-encoding FAS contains nine exons [21], and dominant, heterozygous mutations in the FAS gene cause the above-mentioned ALPS phenotype. These patients show a defect in FAS-mediated apoptosis in lymphocytes and a pathological expansion of double negative T-cells expressing an αβ T-cell receptor [22–24]. Impairment of lymphocyte apoptosis, in general, underlies a variety of autoimmune phenomena [22, 25, 26] and predisposes to diverse lymphomas [26]. FAS mutation itself has also been suggested as contributing factor in the etiology of other diseases including autoimmune phenomena [23, 27–37] as well as malignant lymphomas [36] and solid tumors [38]. Several studies described single nucleotide polymorphisms (SNPs) of the FAS gene to be associated with susceptibility to autoimmune diseases [39–45] as well as cancer [46].

CTLA-4 is a negative regulator of T-cell activation [47] which interacts with its ligands CD80/86 and competes—albeit with a much higher affinity—against CD28 [48, 49]. The CTLA-4 gene has been a primary candidate for a genetic susceptibility to autoimmune diseases [50–54] and to a certain extent to non-Hodgkin’s lymphomas [55]. Furthermore, there are indications for a role of CTLA-4 promoter variants in cancer in general [56], and, additionally, one particular polymorphism in the promoter region has been shown to affect the gene expression level of CTLA-4 [57].

SNPs, themselves, do not cause diseases, but they can help to determine the likelihood that someone will develop a particular disease. Most SNPs are silent, i.e., they do not exert a discernible effect on gene function or phenotype. They can, however, have important consequences for the individual susceptibility to a certain disease or to reactions to certain pharmaceuticals. In addition to changes in single genes that affect disease risk, it is thought that particular combinations of SNPs located across multiple genes contribute to a predisposition for developing a certain disease [58]. Allelic variations in promoter regions could potentially affect the gene expression quantitatively or qualitatively by altering transcription factor binding sites or other regulatory domains.

Given that AILT is frequently associated with autoimmune phenomena, and given that the tumor cells of AILT show an effector phenotype but—despite their expression of FAS and CTLA-4—fail to undergo apoptosis, we investigated whether polymorphisms of the FAS and CTLA-4 genes may be responsible for these features.

Materials and methods

Subjects and SNPs

We selected 53 AILT and 41 PTCL-NOS cases from our archives based on the availability of frozen lymph node specimens or peripheral blood lymphocytes. All cases had been diagnosed according to the World Health Organization classification [1] and were characterized by an extensive immunohistochemical marker panel. All of these 94 lymphomas were analyzed for the presence of the five CTLA-4 gene polymorphisms (see below). As controls, we used data of 173 healthy blood donors that were published previously [54].

In addition, a subset of tumors (ten AILT and ten PTCL-NOS cases) was selected randomly for the analysis of the 29 FAS gene polymorphisms and three mutations (see below). As a control cohort, we used the data population PDR90 (NCBI Single Nucleotide Polymorphism Database,
which comprises SNP information in a global population of 90 individuals. To avoid false positive results due to major differences in sample numbers, ten individuals were selected randomly from this database using the "Random Function" in MS Excel. Some of the examined SNPs or mutations were not included in the PDR90 study; thus, control data were obtained from the literature (see references in Table 1). As a general approach, we preferentially chose SNPs which had already been described in correlation with relevant diseases (Table 1). Furthermore, we included one additional SNP that was detected during our sequence analyses but had not been cited in the literature previously. We compared allelic frequencies between AILT, PTCL-NOS, and healthy control samples for all 29 FAS SNPs and three mutations as well as the genotypes for 20 of these SNPs for which control data was available in the dbSNP population PDR90.

DNA extraction and genotyping

Genomic DNA was extracted from frozen lymph node tissue or from peripheral blood lymphocytes using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

29 FAS SNPs and three mutations (Table 1) were analyzed by polymerase chain reaction (PCR) amplification of the exon and intron sections or the promoter and 3′ UTR regions, respectively. PCR products were sequenced and compared to Genbank accession numbers D31968.1 and AY450925. PCR- and sequencing-primers used were either according to the indicated literature or newly designed (Table 1). The DNA template, 50 ng in a final volume of 25 μl, was amplified in a reaction mixture containing a final concentration of 0.5 μM primer, 0.2 mM dNTPs (Fermentas, St.Leon-Rot, Germany), and 1× Taq polymerase buffer with 1.5 mM MgCl₂ and 0.5 U Taq polymerase (Taq DNA polymerase, recombinant, Invitrogen, Karlsruhe, Germany). PCR reactions were run for 35 cycles in a thermocycler.
| Allele | Count AILT | Count PTCL-NOS | Count Controls | Significance | p AILT/PTCL-NOS | p Co/AILT | p Co/PTCL-NOS |
|--------|------------|---------------|---------------|-------------|----------------|-----------|---------------|
| −1377g>a | 18 | 10 | 20 | 38 | 0.1286 | 0.71 | 0.71 |
| g-1221C>G | C | 20 | 100 | 0 | 0 | n.p. | n.p. | n.p. |
| g-1194A>T | A | 20 | 100 | 0 | 0 | n.p. | n.p. | n.p. |
| −691>c | T | 20 | 100 | 0 | 0 | n.p. | n.p. | n.p. |
| −671a>g | A | 10 | 50 | 10 | 50 | n.p. | n.p. | n.p. |
| g-397C>T | C | 20 | 100 | 0 | 0 | n.p. | n.p. | n.p. |
| g-295Ains | mu | 0 | 0 | 20 | 100 | 0 | 0 | 20 | 100 | n.p. | n.p. | n.p. |
| g-129Cins | mu | 0 | 0 | 20 | 100 | 0 | 0 | 20 | 100 | n.p. | n.p. | n.p. |
| c.161A>G | A | 20 | 100 | 0 | 0 | 0 | 292 | 98.65 | 4 | 1.351 | n.p. | 0.601 | 0.601 |
| c.240G>A | G | 20 | 100 | 0 | 0 | 0 | 20 | 100 | 0 | 0 | n.p. | n.p. | n.p. |
| c.297T>C | T | 20 | 100 | 0 | 0 | 0 | 296 | 100 | 0 | 0 | n.p. | n.p. | n.p. |
| c.335G>A | G | 20 | 100 | 0 | 0 | 0 | 20 | 100 | 0 | 0 | n.p. | n.p. | n.p. |
| c.377G>A | G | 20 | 100 | 0 | 0 | 18 | 90 | 1 | 5 | 0.147 | 0.311 | 0.548 |
| IVS2+176C>T | C | 11 | 55 | 9 | 45 | 14 | 70 | 6 | 30 | 11 | 55 | 9 | 45 | n.p. | 0.601 | 0.601 |
| c.416A>G | A | 19 | 95 | 1 | 5 | 17 | 85 | 3 | 15 | 17 | 85 | 3 | 15 | 0.29 | 0.292 | 1 |
| g.435Gdel | mu | 0 | 0 | 20 | 100 | 0 | 0 | 20 | 100 | 0 | 20 | 100 | 0 | 0 | 20 | 100 | n.p. | 0.147 | 0.147 |
| c.528(+46)c>t | C | 18 | 90 | 2 | 10 | 19 | 95 | 1 | 5 | 18 | 90 | 2 | 10 | 0.548 | 1 | 0.548 |
| c.528(+80)g>c | G | 20 | 100 | 0 | 0 | 20 | 100 | 0 | 0 | 20 | 100 | 0 | 0 | n.p. | n.p. | n.p. |
| c.559C>T | C | 20 | 100 | 0 | 0 | 20 | 100 | 0 | 0 | 20 | 100 | 0 | 0 | n.p. | n.p. | n.p. |
| c.563G>A | G | 20 | 100 | 0 | 0 | 18 | 90 | 2 | 10 | 19 | 95 | 1 | 5 | 0.147 | 0.311 | 0.548 |
| IVS4+699T>C | C | 13 | 65 | 7 | 35 | 14 | 70 | 6 | 30 | 15 | 75 | 5 | 25 | 0.736 | 0.49 | 0.723 |

Table 2 Allelic frequencies of SNPs and mutations in the human FAS gene
After 3 min denaturation at 94°C, each cycle consisted of 45 s at 94°C, 30 s at the assigned annealing temperature and 1 min 30 s at 72°C followed by a final extension step of 10 min at 72°C. PCR products were purified (QIAquick PCR purification kit Qiagen, Hilden, Germany) and aliquots of 7 μl were used for sequencing analysis with 1 μM of the respective primer and 2 μl of BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in a final volume of 10 μl. Samples were analyzed in a 3130×1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

We investigated 6 promoter region-, 13 exon-, 10 intron- and three 3′UTR-SNPs. Sequencing peaks at the location of a SNP showed either one peak indicating homozygosity or two equal lower peaks indicating heterozygosity.

The CTLA-4 SNP genotypes and allele frequencies of three promoter-SNPs (−1722T/C, −1661A/G, and −318C/T), one exon-SNP (+49A/G), and one 3′UTR-SNP (CT60A/G) were identified following previously described polymerase chain reaction or restriction fragment length polymorphism protocols [53, 59, 60].

Distant nomenclature of SNPs including their annotation is used because polymorphisms were strictly named according to the cited literature.

### Statistical analysis

Allele frequencies were compared with the chi-square test. The genotype frequencies between different groups of subjects were compared with chi-square correlation calculated on 2×2 contingency tables. A p value less than 0.05 was considered statistically significant. Staining signal intensity was statistically compared with Spearman rank correlation to diagnoses or SNP haplotype on 2×2 contingency tables.

SNPs with minor allele frequencies ≥5% were estimated by the expectation maximization algorithm as determined by the Haploview program [62]. Logarithm of the odds ratio scores between 2.2 and 3.6 were considered suggestive [63].

### Results

#### FAS gene polymorphisms

Altogether, we analyzed 29 SNPs and three mutations in the coding and flanking regions of the FAS gene (six promoter-
13 exon-, ten intron-, three 3'UTR-SNPs; Table 1). One of the SNPs (IVS4+699T>C) was discovered during our evaluation of the data and was found in the dbSNP database; however, this SNP has not been described in the literature before. Overall, we could not detect SNPs at FA S splice sites among our sequences.

Three gene loci (−691t>c, IVS4+699T>C, IVS7+312C>T) showed differences in allelic frequencies when AILT and PTCL-NOS cases were compared; however, these SNPs did not differ statistically in their allelic frequency values when compared to the control cases. For four SNPs (IVS2+176C>T, IVS4+699T>C, A/T(735)G/C, c.699(+82)c>g), we were able to detect differences in their genotype frequencies when all three groups were compared (Tables 2 and 3, P values in bold type).

Cluster analysis of all three groups for the combined genotypes of the previously mentioned 20 SNPs revealed one subgroup with a high degree of similarities which contained 7/
10 of the AILT cases (Fig. 1). The remaining three AILT cases were more closely linked to PTCL-NOS or control cases.

To detect a possible effect of the \(FAS\) SNP in the promoter region \((-691\text{t>c})\) on gene expression in AILT cases, FAS-staining was performed in 10/10 PTCL-NOS and 9/10 AILT. In general, all AILT tumors showed a significantly stronger FAS expression compared to most of the PTCL-NOS (Table 4 and Fig. 2). Performing cluster analysis of FAS expression and the \(-691\text{t>c} \) genotype, all AILT and two PTCL-NOS cases shared the same combination of parameters (data not shown).

Because complex disease phenotypes including autoimmunity may be influenced by polymorphisms at multiple gene loci presumably related to expression levels or the ability of variant protein domains to interact with functional partners or substrates, we searched for a potential risk related to haplotypic differences by performing haplotype and linkage disequilibrium (LD) analysis. We found LD among several SNPs with minor allele frequency \(\geq5\%\) (data not shown). However, no haplotype blocks emerged.

**CTLA-4** gene polymorphisms

We found no significant association of any of the studied **CTLA-4** SNPs with AILT, PTCL-NOS, and control individuals comparing allelic frequencies or genotypes (Tables 5 and 6). Likewise, cluster analysis of AILT, PTCL-NOS, and controls for the combined genotypes of the **CTLA-4** SNPs revealed no subgroups (data not shown).

The eight AILT cases that could be investigated showed a significantly stronger CTLA-4 expression on the protein level compared to the six PTCL-NOS cases investigated (Table 4 and Fig. 2). Performing cluster analysis of CTLA-4 gene polymorphisms

| Case no | Diagnosis | FAS | CTLA-4 |
|---------|-----------|-----|--------|
| 1       | PTCL-NOS  | +   | n.p.   |
| 2       |           | -   | -      |
| 3       |           | -   | -      |
| 4       |           | +   | -      |
| 5       |           | -   | n.p.   |
| 6       |           | -   | -      |
| 7       |           | +   | -      |
| 8       |           | -   | n.p.   |
| 9       |           | -   | n.p.   |
| 10      |           | -   | -      |
| 11      | AILT      | +   | -      |
| 12      |           | +   | -      |
| 13      |           | +   | -      |
| 14      |           | +   | +      |
| 15      |           | +   | n.p.   |
| 16      |           | +   | +      |
| 17      |           | +   | +      |
| 18      |           | +   | +      |
| 19      |           | +   | +      |

\[p\text{-value} \quad 0.00045 \quad 0.013\]

\(n.p.\) Not possible because of technical reasons
4 expression and the three *CTLA-4* promoter SNP genotypes, no subgroups were detected that included a significant subset of one of the lymphoma entities. Comparing *CTLA-4* expression with the cluster analysis performed on the basis of all *CTLA-4* genotypes and all cases, a completely heterogeneous pattern emerged (data not shown).

**Discussion**

Tumor cells of AILT show an effector phenotype, which is—in the non-malignant counterpart—associated with a propensity to undergo apoptosis. In view of the findings that tumor cells in AILT patients highly express the proapoptotic molecules FAS and *CTLA-4* and are negative for BCL2, a potent inhibitor of apoptosis, we wondered if certain SNPs and/or mutations of the *FAS* and *CTLA-4* genes may be associated with the failure of the tumor cells to undergo apoptosis. However, no such associations could be uncovered in this study. Although six SNPs of the *FAS* gene showed a differential distribution between the AILT and PTCL-NOS subgroups, none of the SNPs appeared to be statistically enriched in comparison to a normal control population. These six SNPs include the SNP −691T>C, which is located in the promoter region of the *FAS* gene and IVS2+176C>T, which is a silent SNP in exon 2. The remaining four SNPs are located in various introns of the *FAS* gene. Comparing these results with data from the literature, three SNPs were associated with certain autoimmune diseases, such as multiple sclerosis (A/T(735)G/C) or Sjögren’s syndrome (c.699(+82)C>G and IVS2+176C>T) in previous reports [42, 43].

To investigate whether the SNP −691T>C located in the promoter region could potentially lead to a modified transcription factor binding site and therefore be of relevance for the expression level of the *FAS* gene, we analyzed the FAS expression of the neoplastic T-cells in AILT and PTCL-NOS by immunohistochemistry. As shown in Table 4 and in the representative images of Fig. 2, FAS staining in AILT and PTCL-NOS cases revealed a significant difference with a significantly higher expression of FAS among AILT cases. Moreover, cluster analysis including only the status of the promoter SNP and the expression data showed a correlation between this SNP and the FAS expression in AILT, with all AILT showing the same
genotype linked to the expression pattern, while PTCL-NOS cases revealed a heterogeneous pattern regarding genotype and FAS expression. Interestingly, two of the three FAS+ PTCL-NOS cases shared the genotype characteristic of AILT, while five PTCL-NOS cases with the same genotype were FAS−. In contrast, the remaining FAS+ PTCL-NOS case revealed a completely different genotype.

We next addressed the question if the promoter SNP −691T>C could lead to changes in transcription factor binding sites. Because no reports exist in the literature to date, we compared the respective sequences with a transcription factor database (http://www.cbrc.jp/research/db/TFSEARCH.html). Potential binding sites were found for AML-1a with a score of 83.4 for allele T and for c-Myc with a score of 80.9 for binding only allele C, which could allow the speculation that in AILT, c-Myc does not interact with this location.

Are the quantitative differences in FAS expression between AILT and PTCL-NOS subgroups attributable to underlying differences in SNP genotypes? We believe that this scenario is highly unlikely. First, SNP distributions in AILT and PTCL-NOS cases did not show statistically significant differences to their occurrence in normal controls. Second, tumor cells in AILT and PTCL-NOS correspond to different subsets of normal T-cell populations [2], which show physiological variations in their FAS expression. Therefore, the difference in FAS expression likely only reflects the distinct expression levels of the physiological counterparts. Future studies will, therefore, have to address the exact quantification of the FAS expression across various physiological differentiation states of T-cells. In addition, DNA-binding studies with AML-1a and c-Myc and the different promoter variants could shed light on the question if and to what extent these transcription factors may play a role in the regulation of FAS expression.

Because certain SNPs in the CTLA-4 gene can lead to functional changes, such as modified promoter activity and lower CTLA-4 surface expression, inefficient processing led to reduced control of T-cell proliferation and a reduced soluble CTLA-4 isoform [51, 57, 64, 65], and because some of these SNPs have been associated with a broad variety of autoimmune diseases [66], we studied CTLA-4 SNPs in AILT and PTCL-NOS cases. However, the statistical evaluation of allelic frequencies and genotypes yielded no correlation between the presence of a CTLA-4 SNP with either the AILT or PTCL-NOS subgroup. Additionally, cluster analysis of genotypes also revealed no subgroups. Even though CTLA-4

### Table 5

| Allele count and frequencies (%) | Significance p |
|----------------------------------|---------------|
| AILT n=53                        | PTCL-NOS n=41 | Controls n=173 |
| −1722T>C                        |               |
| T                                | C            | T    | C    | T    | C    | T    | C    | 0.85 |
|.otally 14=0.83   0.98 | 99 93 7 7 76 93 6 7 321 93 25 7 |
| −1661A>G                        |               |
| A                                | G            | A    | G    | A    | G    | A    | G    | 0.62 |
| 89 84 17 16 71 87 11 13 278 80 68 20 |
| −318C>T                        |               |
| C                                | T            | C    | T    | C    | T    | C    | T    | 0.53 |
| 94 89 12 11 75 91 7 9 309 89 37 11 |
| +49A>G                         |               |
| A                                | G            | A    | G    | A    | G    | A    | G    | 0.52 |
| 61 58 45 42 51 62 31 38 221 64 125 36 |
| CT60A>G                        |               |
| A                                | G            | A    | G    | A    | G    | A    | G    | 0.52 |
| 58 55 48 45 41 50 41 50 181 52 165 48 |

### Table 6

| Genotypes of SNPs in the CTLA-4 gene |
|--------------------------------------|
| Allele count | AILT n=53 | PTCL-NOS n=41 | Controls n=173 |
| −1722T>C     | TT 46 7 0 36 4 1 | TT 36 4 1 150 21 2 | 0.83 |
| −1661A>G     | AA 36 17 0 30 11 0 | AA 30 11 0 111 56 6 | 0.40 |
| −318C>T      | CC 43 8 2 35 5 1 | CC 35 5 1 140 29 4 | 0.93 |
| +49A>G       | AA 21 19 13 16 19 6 | AA 16 19 6 78 65 30 | 0.60 |
| CT60A>G      | AA 14 20 19 10 21 10 | AA 10 21 10 35 95 43 | 0.28 |
expression significantly differed between AILT and PTCL-NOS cases, this again is likely to be explained by the distinct differentiation states of the normal counterparts of the neoplastic populations in AILT and PTCL-NOS. This is supported by comparing CTLA-4 expression to promoter SNP genotypes and the cluster analysis of all five genotypes, which did not show any correlation.

Interestingly, in another study, a difference in the occurrence of the +49AA genotype of the CTLA-4 gene was described between non-Hodgkin’s lymphoma patients and controls [55]. Four of 44 cases in this study were T-cell lymphomas, but, unfortunately, the authors did not provide detailed information on this subset. Thus, results between our and the previously mentioned study cannot be compared at the present time.

In summary, our study provides evidence that SNPs or mutations of the FAS and CTLA-4 genes are not responsible for the failure of the tumor cells in AILT patients to undergo apoptosis and for the accompanying autoimmune phenomena. Future studies will have to unravel the mechanism by which the neoplastic population in AILT that appears to have an effector T-cell phenotype and is equipped with high expression of the pro-apoptotic molecules FAS and CTLA-4 circumvent the induction of cell death.

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Ethical standards  Ethics approval for the entire study was obtained from the ethics committee, Medical Faculty, University of Würzburg, Germany. In general, the procedures followed the Helsinki Declaration of 1975. Informed patient consent was not required by the ethics committee because diagnostic specimens can be used for research purposes after anonymization.

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