Inactivation of Tumor Suppressor Genes $p15^{INK4b}$ and $p16^{INK4a}$ in Primary Cutaneous B Cell Lymphoma

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Primary cutaneous B cell lymphomas represent a distinct group of lymphoproliferative disorders that can be distinguished from systemic lymphoma by their good response to local treatment and favorable prognosis. In systemic B cell lymphoma, inactivation of $p15^{INK4b}$ and $p16^{INK4a}$ is frequently observed and may be associated with a poor prognosis. There have been no comprehensive studies in primary cutaneous B cell lymphomas, however. Mechanisms of p15/p16 inactivation include loss of heterozygosity, homozygous deletion, promotor region hypermethylation, and point mutation. We analyzed DNA from 36 cases of primary cutaneous B cell lymphomas, four systemic B cell lymphomas, and six benign B cell lymphoproliferative infiltrates for abnormalities of p15 and p16 using microsatellite markers for 9p21, methylation specific polymerase chain reaction, and polymerase chain reaction/single stranded conformational polymorphism analysis with exon specific primers. Expression of both p15 and p16 protein was assessed by immunohistochemistry. Loss of heterozygosity at 9p21 was identified in 2 out of 36 primary cutaneous B cell lymphomas. Hypermethylation of p15 and p16 promotor regions was identified in 8 of 35 (23%) and 15 of 35 (43%) cases, respectively. In two cases p16 hypermethylation was identified in recurrent disease but not in the initial tumor. No point mutations were identified. In seven patients, however, a polymorphism was observed in exon 3 of the p16 gene. In primary cutaneous B cell lymphomas with allelic loss or promotor hypermethylation of either p15 or p16, loss of expression in tumor cells was identified in 5 of 8 and 9 of 10 cases, respectively. Our findings suggest that $p15^{INK4b}$ and $p16^{INK4a}$ biallelic gene abnormalities are common in primary cutaneous B cell lymphomas, most frequently as a result of promotor hypermethylation. The presence of abnormalities in recurrent disease in some cases suggests that inactivation of p15 and p16 may be involved in disease progression. ***Key words: chromosome 9/loss of heterozygosity/promotor hypermethylation. J Invest Dermatol 118:941–948, 2002***

Primary cutaneous B cell lymphomas (PCBCL) represent a heterogeneous group of diseases with different clinical, histologic, and immunophenotypic features and may be either indolent or aggressive. Specific chromosomal abnormalities are characteristic of different systemic lymphomas but attempts to identify tumor-specific chromosomal abnormalities in PCBCL have been disappointing. The t(14; 18) translocation is found in the majority of nodal follicular lymphomas, leading to upregulation of the *bcl-2* gene, which prevents apoptosis (Weiss *et al*, 1987). This translocation is not usually found in PCBCL even in those with histologic features suggestive of a follicle center cell origin (Cerroni *et al*, 1994; Grønbæk *et al*, 2000; Child *et al*, 2001*a*; Franco *et al*, 2001).

Most malignancies accumulate a series of genetic abnormalities including inactivation of different tumor suppressor genes. Biallelic gene inactivation may be a consequence of different mechanisms including loss of chromosomal material [loss of heterozygosity (LOH) or homozygous deletion], intragenic mutation, and/or hypermethylation of CpG islands within the promotor leading to transcriptional silencing (Jones and Laird, 1999).

The tumor suppressor genes $p15^{INK4b}$ and $p16^{INK4b}$ are located on chromosome 9p21 and encode cyclin-dependent kinase inhibitors (negative cell cycle regulators) (Serrano *et al*, 1993; Kamb *et al*, 1994). Both proteins bind to cyclin-dependent kinases 4 and 6 (CDK4, CDK6) and inhibit the kinase activity of the cyclin-kinase complexes, producing functional inactivation of the retinoblastoma protein and the subsequent block of the G1 phase of the cell cycle.

Loss of cell cycle control is a common alteration in neoplasia (Hirama and Koeffler, 1995), leading to uncontrolled cell growth. Inactivation of both p15 and p16 by homozygous deletions, mutation, and hypermethylation occurs in a wide range of human tumors, and has been identified in systemic B cell lymphoma (Dreyling *et al*, 1995; Gombart *et al*, 1995; Herman *et al*, 1997; Elenitoba-Johnson *et al*, 1998; Baur *et al*, 1999). p15 and p16 gene inactivation is associated with aggressive tumors and may play a role in progression of indolent disease (Herman *et al*, 1997; Elenitoba-Johnson *et al*, 1998; Pinyol *et al*, 1998; Villuendas *et al*, 1998).

We have analyzed 36 PCBCL and four cases of systemic lymphoma with cutaneous lesions for inactivation of both p15 and p16 genes by various mechanisms including LOH, promotor

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Abbreviations: CHOP, cyclophosphamide, adriamycin, vincristine, prednisolone; DLCL, diffuse large cell lymphoma; FCCL, follicle center cell lymphoma; LOH, loss of heterozygosity; MZL, marginal zone lymphoma; PCBCL, primary cutaneous B cell lymphoma.

hypermethylation, and point mutation. We have correlated these data with immunohistochemical evidence of p15 and p16 expression and the histologic subtype and grade of tumor.

MATERIALS AND METHODS

Frozen tissue samples from 36 cases of PCBCL, four systemic follicular lymphomas with cutaneous lesions, and six benign cutaneous B cell infiltrates were retrieved. In three cases of PCBCL, recurrent lesions were also available for analysis. The corresponding histologic and immunohistochemistry sections had been reviewed and classified according to the World Health Organization Classification of Tumors of Haematopoietic and Lymphoid Tissues (Harris, 2001). Cases included eight follicle center cell lymphoma (FCCL), 19 marginal zone lymphoma (MZL), eight diffuse large cell lymphoma (DLCL), and one unclassifiable case.

All cases of PCBCL studied had either a dominant clonal immunoglobulin heavy chain gene (IgH) rearrangement in lesional skin, identified using polymerase chain reaction (PCR) methodology (Child *et al*, 2001*b*), or immunohistochemical evidence of light chain restriction. For each case, a corresponding polyclonal control sample was also obtained. Tumor cells made up > 50% of the infiltrate in 22 cases and > 75% in eight cases (all DLCL). In six cases a B cell clone was identified by gene analysis but the admixture of inflammatory cells made up > 50% of the infiltrate.

DNA extraction DNA was extracted from freshly obtained or frozen tissue specimens according to standard procedures using proteinase K digestion and phenol/chloroform extraction. For promotor hypermethylation analysis, a control DNA sample was extracted from the Raji cell line (ECCAC No: 85011429) (derived from a case of Burkitt's lymphoma, which is methylated at the promotor CpG islands of both $p16^{INK4a}$ and $p15^{INK4b}$).

Analysis of allelic loss on 9p21 DNA from both lesional skin and normal tissue in each case was analyzed for LOH by PCR amplification of the following highly polymorphic dinucleotide repeat sequences: D9S126, IFNA (a marker located within the interferon-α locus flanking the p15 and p16 genes), intra p15/p16 (a dinucleotide repeat located between the $p15^{INK4b}$ and $p16^{INK4a}$ genes), and a further marker, D9S171, also flanking the p15 and p16 genes (Amersham Pharmacia Biotech, Little Chalfont, U.K.) (Heyman *et al*, 1996). The reaction mix (20 μl final volume) consisted of 0.2–0.5 μg of template DNA, 1 × PCR buffer (Amersham Pharmacia Biotech), 200 μmol per liter [α^{33}P] deoxycytidine triphosphate, 200 μmol per liter each of the other deoxyribonucleoside triphosphates, 0.1% Tween, 100 ng synthetic oligonucleotide primers (Amersham Pharmacia Biotech), and 1 unit of *taq (thermus aquaticus)* polymerase (Amersham Pharmacia Biotech) in combination with *taq* antibody (Clontech, Basingstoke, U.K.).

The presence of homozygous deletions was also assessed in all cases by a comparative multiplex PCR assay (Cairns *et al*, 1994) using a primer set (D13S160) from outside the area of 9p21 and a primer set for the locus of suspected homozygous deletion on 9p21 (D9S1747 and D9S1748).

PCR was performed using a Perkin Elmer thermal cycler (Model 9700, Perkin Elmer, Warrington, U.K.). The annealing temperatures used were as follows: D9S126, IFNA, and D9S171, D9S1747, D9S1748, 55°C; intra p15/p16, 50°C. The PCR were performed over 25–30 cycles.

$P15^{INK4b}$ and $p16^{INK4a}$ promotor region hypermethylation The methylation status of the p15 and p16 gene promoters was analyzed using bisulfite modification. Methylation specific PCR was carried out as described by Herman *et al* (1996). In this technique, DNA is chemically modified by sodium bisulfite, which changes the unmethylated but not the methylated cytosines into uracil. The bisulfite-treated DNA is subjected to PCR amplification using primers designed to anneal specifically to the methylated and unmethylated bisulfite-modified DNA within a given gene. A PCR product is obtained when the sequence covered by the primers is methylated. Briefly, 1 μg genomic DNA was denatured in 0.2 mol per liter NaOH (Sigma-Aldrich, Poole, U.K.) (volume 50 μl) for 20 min at 37°C. After the addition of 550 μl of a freshly prepared solution containing 10 mmol per liter hydroquinone (Sigma-Aldrich) and 3 mol per liter sodium bisulfite (Sigma-Aldrich) (pH 5.2), the samples were incubated overnight (16–21 h) at 55°C. The bisulfite-modified DNA was purified on Qiaquick DNA purification columns (Qiagen, Crawley, U.K.) according to the manufacturer's specifications and eluted with 50 μl of water. The DNA was treated with 50 μl of 0.6 mol per liter NaOH for 20 min at room temperature, precipitated by ethanol at –70°C, and resuspended in 20 μl of water. Bisulfite-modified DNA was amplified by PCR using primer sets specific for the methylated and unmethylated p15 (p15 M and U) and p16 (p16 M and M2, U and U2) sequences (Herman *et al*, 1996). Prior to chemical modification, PCR with wild-type (W) primers were also performed using nonbisulfite-modified DNA to ensure adequate DNA quality. Because tumor cells are always admixed with reactive cells in lymphomas, the PCR amplifications using primers specific for unmethylated DNA were considered as positive controls for the efficiency of chemical modification. The reaction mix (50 μl final volume) consisted of 1 μg of template DNA, 1 × PCR buffer (Amersham Pharmacia Biotech), 200 μmol per liter [α^{33}P] deoxycytidine triphosphate, 200 μmol per liter each of the other deoxyribonucleoside triphosphates, 2.5 mmol per liter $MgCl_2$, 0.1% Tween, 100 ng synthetic oligonucleotide primers (Amersham Pharmacia Biotech), and 1 unit of *taq (thermus aquaticus)* polymerase (Amersham Pharmacia Biotech) in combination with *taq* antibody (Clontech). The annealing temperatures used were as follows: p16 M and M2, 65°C; p16 U and U2, 60°C; p15 M and U, 60°C. The PCR were performed over 30–35 cycles.

Point mutations and small deletions Genomic DNA was subjected to PCR amplification using primers for exons 1–3 of the p16 gene and exons 1–2 of the p15 gene (Heyman *et al*, 1996). The reaction mix was similar to that used for LOH analysis, but amplification of p16 exons 2 and 3 and p15 exon 2 required additional $MgCl_2$ at a concentration of 2.5 mmol per liter. The annealing temperatures used were as follows: p16 exon 1, 57°C; p16 exon 2 and 3, 60°C; p15 exon 1, 55°C; p15 exon 2, 60°C. All PCR were performed over 35 cycles.

Analysis of amplification products

LOH analysis and promotor hypermethylation An aliquot of the radiolabeled PCR products was run on a denaturing polyacrylamide gel [6% acrylamide (Gibco BRL, Paisley, U.K.), 7 mol per liter urea (United States Biochemicals, Cleveland, OH)]. The gel was run at 30 W for 2–4 h, depending on the size of the PCR product, at room temperature in 0.5 × TBE buffer. For LOH analysis, each sample was run in a pair with a corresponding normal control sample from each patient. For promotor hypermethylation, the amplification products using the methylated and unmethylated primers were run next to each other for each patient with the Raji cell line as a positive control.

Comparative multiplex PCR An aliquot of the PCR products was run on a 2% agarose gel. A homozygous deletion would be indicated when the D9S1747 or D9S1748 signal was significantly less than the signal from the D13S160 (control) allele (as assessed with densitometry).

Point mutations/small deletions Radiolabeled products were separated by single stranded conformational polymorphism (SSCP) analysis polyacrylamide gel (with 10% glycerol) electrophoresis run at 30 W for 8 h at 4°C.

Gels were transferred to a sheet of 3 mm Whatman paper and vacuum dried at 80°C. Autoradiography was carried out at room temperature for 24–72 h with an intensifying screen.

Sequence analysis Products obtained from promotor hypermethylation of the Raji cell line and similar products obtained from cases of PCBCL were sequenced to confirm identification of the correct product. Abnormal bands obtained from PCR/SSCP analysis were also sequenced to identify mutations/deletions.

Bands were excised from the gels and re-amplified using a biotinylated 5′ methylation specific or 5′ exon specific primer together with the appropriate 3′ primer. Successful re-amplification was determined by electrophoresis on 2% agarose gels and the PCR products were passed through microspin columns (Amersham Pharmacia Biotech) to remove unincorporated primers. The amplification products were immobilized on the surface of streptavidin-coated microbeads (Dynabeads M-280, Dynal, Oslo, Norway) to produce single-stranded PCR products. The chain termination DNA sequencing method was performed using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemicals). Products were analyzed by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

Immunohistochemical analysis for p15 and p16 expression Paraffin-embedded histologic skin sections corresponding to the DNA samples in 31 cases of PCBCL, three cases of systemic FCL, and five cases of reactive lymphoid hyperplasia were identified and stained for both p15 (Laboratory Vision, Newmarket, U.K.) and p16 (Santa Cruz Biotechnology, Santa Cruz) expression using mouse monoclonal antibodies. Both p15 and p16 are nuclear proteins and nuclear staining of cells by the antibody was considered as a positive

signal for protein expression. Sections were dewaxed, incubated in 0.01 M sodium citrate, and microwaved for 18 min. The antibodies were diluted, applied to the sections for 30 min, and detected using the indirect streptavidin technique. The final reaction products were developed with 3′-3″ diaminobenzidine. Sections from reactive lymph nodes were used as controls for normal expression of p15 and p16. Sections were examined by a histopathologist (EC) without prior knowledge of the results obtained from the molecular studies. The percentage of tumor cells with nuclear staining of p15/p16 was recorded as follows: +++, 75%–100% of tumor cells positive; ++, 50%–75% of tumor cells positive; +, 25%–50% of tumor cells positive; +–, < 25% of tumor cells positive.

Correlation with clinical features (summarized in Table I) Adequate follow-up data were available for 34 of the 36 cases of PCBCL investigated. Clinical data collected included age of onset and length of follow-up; site of lesions and whether they were solitary or multiple; number of cutaneous recurrences and those with ongoing disease. In relevant cases, extracutaneous spread and date of death were also recorded.

Overall, 19 males and 17 females were affected. The median age at onset of disease was 54 y (range 12–93 y). The median age of onset in the cases of DLCL was 76 y (range 22–93 y). In the 34 cases with follow-up data the median length of follow-up was 51 mo (range 12–193 mo). The most common site of presentation was the scalp in cases of FCCL, the trunk in MZL, and the lower leg in cases of DLCL. Eleven patients presented with multifocal lesions. Cutaneous recurrence was frequent following treatment and six patients subsequently developed lymph node involvement (four of these cases had DLCL). Three of these six patients have died of their disease (all with DLCL). A further patient with DLCL developed bone marrow involvement and also died. The majority of patients were treated with radiotherapy, three patients were treated with single agent chemotherapy (chlorambucil), and six were given multiagent chemotherapy with CHOP (cyclophosphamide, adriamycin, vincristine, prednisolone) (five of these cases had developed lymph node disease). Two patients with DLCL that had failed to respond to CHOP were treated with rituximab, which produced a short remission; one patient has died and the other received an autologous peripheral blood stem cell transplant, leading to a remission of 18 mo, but this patient has subsequently developed recurrent cutaneous disease.

RESULTS (SUMMARIZED IN TABLE II)

LOH LOH was identified in 2 of 36 (6%) cases of PCBCL. In one case (DLCL) loss was identified with the intragenic p15/p16 marker (intra p15/16) but not with the flanking markers (**Fig 1**).

In the second case (MZL) loss occurred at one flanking marker (IFNA) but was identified only in DNA extracted from a second biopsy at disease recurrence.

No samples demonstrated LOH at both markers flanking either the p15 (p15/p16 and D9S171) or p16 (p15/16 and IFNA) genes, suggesting the absence of homozygous deletions. Larger homozygous deletions affecting both the p15 and the p16 gene could be missed using this method alone, but comparative multiplex PCR also failed to identify large homozygous deletions.

Promotor hypermethylation Promotor hypermethylation at p15 was identified in 8 of 35 (23%) cases of PCBCL overall, consisting of 1 of 8 (12.5%) FCCL, 6 of 18 (33%) MZL, and 1 of 8 (12.5%) DLCL (**Fig 2*a***).

Promotor hypermethylation at p16 was identified in 15 out of 35 (43%) cases of PCBCL overall, consisting of 3 of 8 (37.5%) FCCL, 7 of 18 (39%) MZL, 4 of 8 (50%) DLCL, and the one unclassifiable case (**Fig 2*b***).

It was also identified in two of four cases of systemic FCL. In two cases of PCBCL, p16 hypermethylation was identified only in DNA from biopsies taken at disease recurrence but not in the original biopsies.

Hypermethylation of both p15 and p16 promotors was identified in 4 of 35 (11%) cases (one FCCL, two MZL, one DLCL).

Sequence analysis of methylated and unmethylated p15 and p16 products obtained from the Raji cell line and selected cases confirmed amplification of the correct product.

Of the six benign B cell lymphoproliferative infiltrates that were analyzed, all amplified using the primers specific for unmethylated DNA but none showed promotor hypermethylation at either the p15 or p16 promotor.

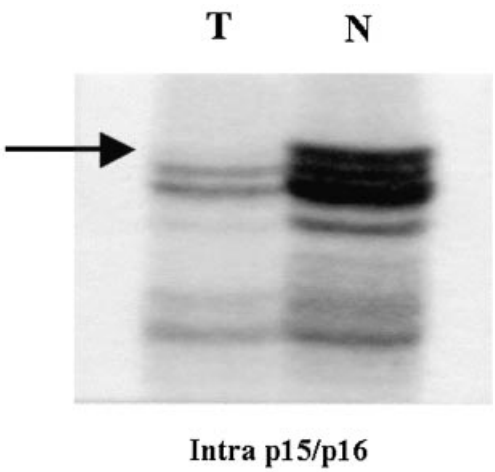

Figure 1. Results of LOH analysis. Polyacrylamide gel electrophoresis of tumor (T) and control (N) DNA PCR products in one case with LOH using primers intra p15/p16. *Arrow* indicates loss of an allele (> 50% reduction of band intensity). Additional bands in both tumor and control samples are present due to DNA slippage, which is more readily apparent when using ^{33}PdCTP than with ^{32}PdCTP radiolabeled products.

In one case the bisulfite-modified DNA failed to amplify with primers specific for both methylated and umethylated p15 and p16 promotors suggesting that modification had failed.

Combined LOH and promotor hypermethylation, suggesting a biallelic event, was not identified in any case, although biallelic promotor hypermethylation cannot be excluded.

PCR/SSCP analysis for point mutations and small deletions Identical band shifts were identified in seven cases when PCR products were analyzed following amplification of exon 3 of the p16 gene (**Fig 3*a***).

Sequence analysis identified a C→T transition at nucleotide position 580, which is a previously described polymorphism in the untranslated region of exon 3 (Holland *et al*, 1995) (**Fig 3*b***).

No point mutations or small deletions were identified in either the p15 or p16 gene.

Correlation with p15 and p16 expression (see Table II) p15 expression by tumor cells in all eight cases of PCBCL with p15 promotor hypermethylation was examined (cases 3, 10, 11, 13, 17–19, 30). Five of eight cases showed decreased expression by tumor cells (positive nuclear staining of only 50%–75% of tumor cells in three cases and 25%–50% in two cases). Of 15 cases of PCBCL with p16 promotor hypermethylation, paraffin-embedded material corresponding to the extracted DNA was available for analysis in only 10 cases (cases 1, 3, 9, 11, 13, 26, 28, 30, 31, 34). Nine of ten demonstrated reduced expression of p16 by tumor cells (< 25% tumor cells expressed p16 in one case, 25%–50% tumor cells expressed p16 in four cases, and between 50% and 75% tumor cells expressed p16 in four cases). In cases 1 and 34, where p16 hypermethylation had been identified in recurrent disease only, absent p16 expression was identified only in histologic sections from recurrent disease but not in the original biopsy material. In case 35, in which LOH between p15 and p16 genes but no other genetic abnormality had been identified, fewer than 50% of the tumor cells expressed p15 and fewer than 25% expressed p16.

Of the 15 cases with no molecular evidence of gene inactivation, immunohistochemistry was performed in 14 cases. Ten cases showed expression of p15 and p16 by all tumor cells. In the other four cases, one showed absent expression of p15, and three showed absent expression of p16 by a subpopulation of tumor cells.

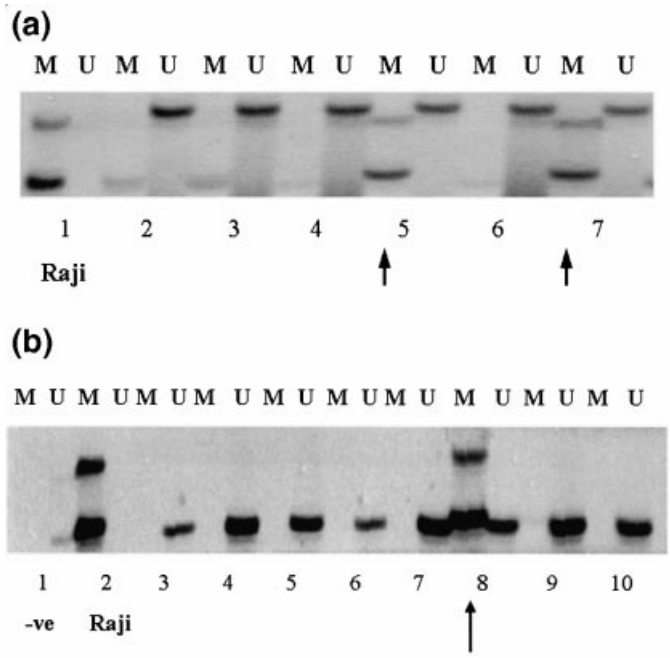

Figure 2. Methylation specific PCR of bisulfite-modified DNA. (*A*) Using primer pairs specific for methylated and unmethylated $p15^{INK4b}$ promotor sequences in each case. M, primers specific for methylated DNA; U, primers specific for unmethylated DNA. 1 is DNA from Raji cell line (positive control) showing amplification with the primer pair specific for methylated DNA. 5 and 7 illustrate p15 hypermethylation in two cases of PCBCL (a product is also obtained with the primers specific for unmethylated DNA because of amplification of DNA from normal reactive cells present in the initial biopsy specimen). 2–4 and 6 show amplification with primers specific for unmethylated DNA only. The two bands obtained in the methylated lanes represent denatured methylated single DNA strands, confirmed by sequence analysis of both products. (*B*) Using primer pairs specific for methylated and unmethylated $p16^{INK4a}$ promotor sequences in each case. M, primers specific for methylated DNA; U, primers specific for unmethylated DNA. 1, negative control (no DNA); 2, DNA from Raji cell line (positive control) showing amplification with the primer sequences specific for methylated DNA but not with those specific for unmethylated DNA. 8 illustrates p16 promotor hypermethylation in one case of PCBCL (a product is also obtained with the primers specific for unmethylated DNA because of amplification of DNA from normal reactive cells present in the initial biopsy specimen). 3–7, 9, and 10 show amplification with primers specific for unmethylated DNA only. The two bands obtained in the methylated lanes represent denatured methylated single DNA strands, confirmed by sequence analysis of both products.

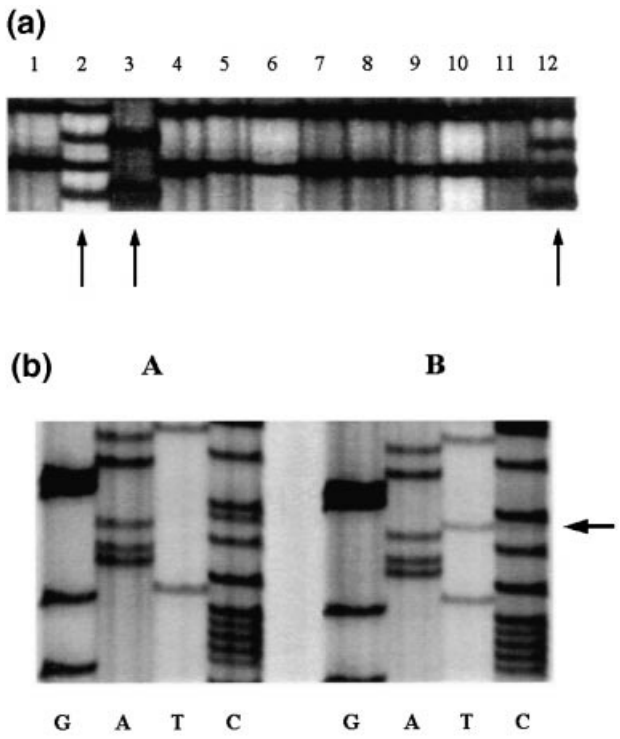

Figure 3. PCR/SSCP and sequence analysis of $p16^{INK4a}$ exon 3. (*A*) PCR/SSCP analysis of $p16^{INK4a}$ exon 3. An identical band shift can be seen in *lanes 2*, *3*, and *12*. (*B*) Sequence analysis of the aberrant band identified a C→T base change at position 580 in the 3′ untranslated region of the p16 gene. *A*: Sequence obtained from a case with a normal pattern on SSCP analysis. *B*: Sequence obtained from a case with a band shift. *Arrow* indicates C→T base change.

In PCBCL, particularly cases of MZL, the tumor population is often relatively small compared with the surrounding infiltrate of reactive cells. In six cases of MZL (cases 10, 11, 14, 16, 18, and 19) the percentage of tumor cells in the infiltrate was less than 50% of the total cell population [as assessed by histology and immunohistochemistry and the intensity (using densitometry) of the clonal B cell population by PCR analysis of the immunoglobulin heavy chain gene]. Absent expression of p15 (case 14) and p16 (case 16) in a subpopulation of tumor cells with no detectable molecular abnormalities could occur because the tumor population is too small for the abnormalities to be identified using the techniques employed.

In the two cases of systemic lymphoma with p16 hypermethylation, absent expression of p16 occurred in between 50% and 75% of tumor cells.

Normal expression of both p15 and p16 in mononuclear cells was seen in the five cases of reactive lymphoid hyperplasia examined.

In all cases, p15 and p16 staining of basal keratinocytes within the epidermis was identified and acted as a positive internal control (**Fig 4*a*, *b*, *c***).

Correlation with clinical features (see Table I) Overall, cases of PCBCL with promotor hypermethylation/LOH had had their disease for a shorter period (median 46 mo) than those without detectable abnormalities (median 70 mo). p15/p16 gene abnormalities were no more frequent in cases with multiple compared with solitary lesions and did not correlate with site of disease or rates of cutaneous recurrence. Six patients subsequently developed lymph node involvement; promotor hypermethylation was identified in three of these patients (all with DLCL). Allelic loss at 9p21 was identified in a further patient with DLCL who remains well following treatment (follow-up 2 y).

The two cases of systemic FCCL with p16 hypermethylation are alive with ongoing, indolent disease.

DISCUSSION

The results from this study suggest that p15 and p16 gene abnormalities at 9p21 are common in PCBCL. Abnormalities were identified in 61% of cases studied and most frequently occurred as a result of promotor hypermethylation (55%), affecting the p16 gene more often than the p15 gene (43% compared to 23%). In the majority of cases with p15 or p16 gene abnormalities it was possible to demonstrate reduced expression of either p15 or p16 protein by tumor cells, which suggests biallelic hypermethylation leading to gene inactivation. In contrast, the small number of cases in which there was normal p15 and p16 protein expression in the context of hypermethylation may be explained by involvement of only one allele.

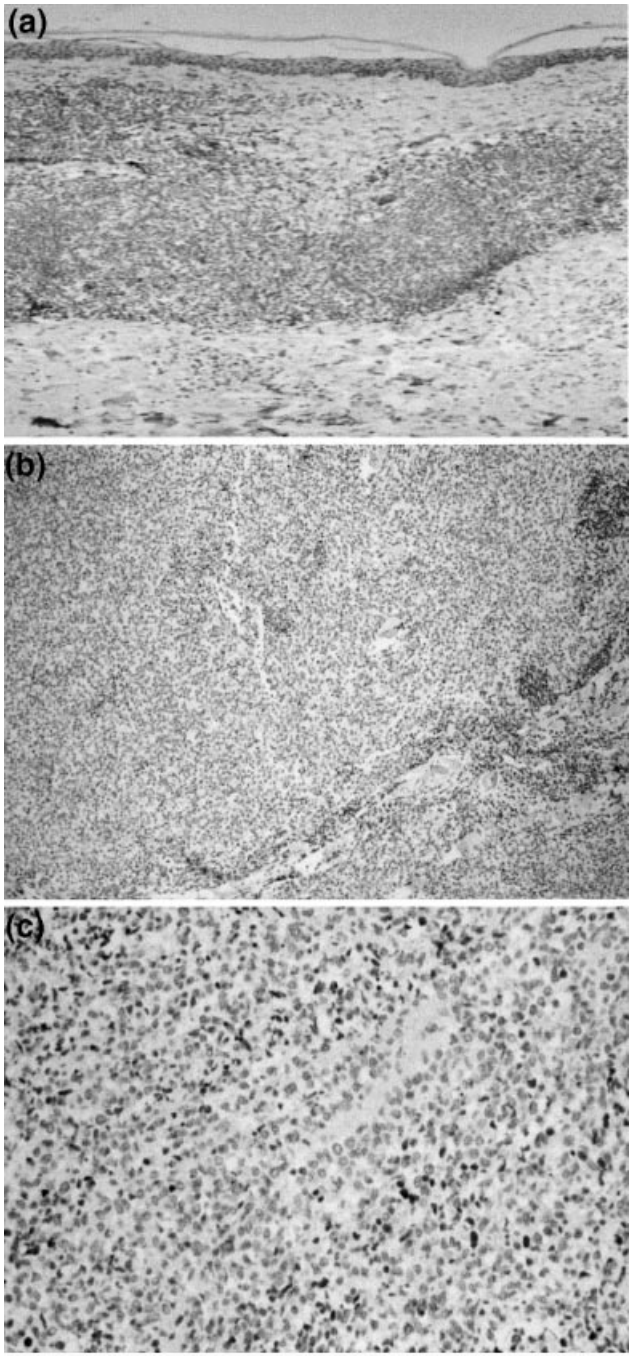

Figure 4.Immunohistochemical evidence of reduced expression of p15 and p16 protein by the neoplastic cells. (*a*) Section from a low-grade MZL with p15 hypermethylation demonstrating expression of p15 by < 50% of tumor cells. Nuclear staining of basal keratinocytes acts as a positive internal control (original magnification ×10). (*b*) Section from a high-grade DLCL with p16 hypermethylation demonstrating absent expression of p16 protein (original magnification ×40). (*c*) Section from a DLCL with LOH identified at intra p15/p16 showing that <25% of the large tumor cells express p16 (original magnification ×100).

High rates of hypermethylation, affecting both the p15 and p16 promotor regions, have been identified in systemic B cell non-Hodgkins lymphoma. In a study by Herman *et al* (1997), hypermethylation of $p16^{INK4a}$, often without alterations of $p15^{INK4b}$, was seen commonly in NHL and was more frequent in cases with high-grade histology. By contrast, Baur *et al* (1999) identified p15 hypermethylation in 64% (55% low-grade, 78% high-grade) and p16 hypermethylation in 32% (low-grade 21%, high-grade 50%) of nodal forms of NHL. Although our data indicate that p15 and p16 hypermethylation occur commonly, p16 hypermethylation is more frequent, particularly in DLCL, and in the cases of systemic NHL that we studied, only p16 hypermethylation was identified. Both p15 and p16 hypermethylation was found in 11% of our cases and was equally distributed between different subtypes.

LOH was an infrequent finding and homozygous deletions were not identified in any case examined. We cannot fully exclude false negative results, however, because, in PCBCL, tumor cells are often accompanied by a significant infiltrate of reactive cells, which can lead to failure to identify LOH in tissue samples. Amplification of DNA extracted from microdissected tumor cells may identify further cases with allelic loss, as has previously been identified in primary cutaneous CD30-positive large cell lymphoma (Böni *et al*, 2000).

Both cases with LOH at 9p21 had high-grade features on histology, in which the majority of cells were neoplastic. LOH implies that one allele of either gene has been deleted, leaving the second allele still functional. Expression of p15 and p16 was normal in one case, but in the case with LOH at the intragenic p15/16 marker, expression of both p15 and p16 by the tumor cells was less than 50% and 25%, respectively. These findings suggest inactivation of the second allele by an alternative mechanism, but in this case no other genetic abnormality was identified by our techniques.

In systemic B cell lymphoma, homozygous deletions at 9p21 are found in only a small proportion of cases and appear to be associated with progression of low-grade follicle center lymphoma to DLCL but not with *de novo* high-grade DLCL (Elenitoba-Johnson *et al*, 1998). In our series, three cases of recurrent disease were studied, and in one case of MZL allelic loss was only identified in DNA from a recurrent lesion, which histologically showed high-grade features; however, no homozygous deletions were identified. In hematologic malignancies homozygous deletions that extend over 500 kb and encompass both p15 and p16 genes and the interferon gene cluster are common (Dreyling *et al*, 1995) and would not be detected using our LOH technique. Comparative multiplex PCR (Cairns *et al*, 1994), however, also failed to detect any homozygous deletions. Southern blot analysis (Ogawa *et al*, 1994) would have helped to identify larger homozygous deletions in our cases.

No point mutations or small deletions were identified, other than a previously recognized polymorphism at position 580 in the 3′ untranslated region of p16 exon 3 (Holland *et al*, 1995) in seven cases. These results are consistent with other studies in NHL (Pinyol *et al*, 1998; Villuendas *et al*, 1998) and suggest that in contrast to other solid organ malignancies such as melanoma (Holland *et al*, 1999) mutations are not an important mechanism of p15/p16 gene inactivation. SSCP/PCR analysis only detects mutations that result in an abnormal electrophoretic pattern, however, and therefore it is possible that other mutations may have been missed.

Studies of p15 and p16 genes in cutaneous T cell lymphoma (CTCL) have also been performed recently. Peris *et al* (1999) showed lack of p16 expression in 25% of cases of mycosis fungoides, but found no association with point mutations or minor deletions of the p16 gene. Other studies have subsequently identified promotor hypermethylation, leading to gene inactivation. Scarisbrick *et al* (2002) showed that hypermethylation in CTCL most commonly involves the p15 promotor rather than the p16 promotor. Other studies have shown p16 hypermethylation more commonly in tumor stage mycosis fungoides (Navas *et al*, 2000), however, suggesting that p16 inactivation is involved in progression of disease. In our study, p16 promotor hypermethylation was more frequent in DLCL, and was identified in three of four patients with DLCL who developed lymph node involvement. p15/p16 gene inactivation was also identified in recurrent but not presenting lesions in

Table I. Clinical details of patients with PCBCL

Case	Disease type	Sex	Age at onset	Original site	Recurrence	Length of disease	Extracutaneous spread	Treatment
1	FCCL	F	53	Scalp	× 3	46 mo	–	Radiotherapy
2	FCCL	M	66	Scalp	× 3	84 mo	–	Radiotherapy
3	FCCL	F	78	Scalp	Nil	54 mo	–	Radiotherapy
4	FCCL	M	76	Scalp	Nil	Lost to F/U	–	Radiotherapy
5	FCCL	F	28	Scalp	Nil	72 mo	–	Radiotherapy, CHOP
6	FCCL	F	31	Multifocal	Ongoing	18 mo	–	Nil
7	FCCL	M	46	Trunk	Nil	15 mo	–	Radiotherapy
8	FCCL	M	47	Scalp	Nil	18 mo	–	Radiotherapy
9	MZL	M	51	Scalp	Nil	32 mo	–	Radiotherapy
10	MZL	F	74	Multifocal (trunk)	Ongoing	36 mo	–	Declined treatment
11	MZL	F	60	Multifocal (face, trunk)	Ongoing	60 mo	–	Radiotherapy
12	MZL	M	63	Face, trunk	Multiple	138 mo	Lymph nodes (at 108 mo)	Radiotherapy, CHOP
13	MZL	M	54	Face, trunk	Multiple	78 mo	–	Radiotherapy
14	MZL	M	46	Trunk	× 2	84 mo	–	Radiotherapy
15	MZL	M	45	Trunk	× 2	69 mo	Lymph node	Radiotherapy, CHOP
16	MZL	F	61	Upper limbs	Nil	60 mo	–	Radiotherapy
17	MZL	M	33	Scalp	× 1	24 mo	–	Excision, radiotherapy
18	MZL	F	64	Trunk	Ongoing	120 mo	–	Radiotherapy, chlorambucil
19	MZL	M	42	Multifocal (trunk)	Ongoing	193 mo	–	Radiotherapy, s/c interferon-α
20	MZL	M	41	Trunk	Multiple	78 mo	–	Radiotherapy
21	MZL	F	59	Arm, forehead	Nil	48 mo	–	Radiotherapy
22	MZL	M	28	Trunk	Ongoing	126 mo	–	OHchloroquine
23	MZL	F	58	Upper limbs	Nil	36 mo	–	Excision
24	MZL	F	70	Face	Nil	24 mo	–	Radiotherapy
25	MZL	M	27	Multifocal	Ongoing	150 mo	–	Radiotherapy
26	MZL	M	35	Multifocal	Ongoing	55 mo	–	Radiotherapy
27	MZL	M	12	Trunk, arm	× 1	88 mo	–	Radiotherapy
28	Unclassifiable	M	40	Trunk	Lost to F/U	Lost to F/U	Lost to F/U	Lost to F/U
29	DLCL	F	22	Upper and lower limbs	Multiple	138 mo	Lymph node (60 mo)	Radiotherapy, rituximab, CHOP, APBSCT
30	DLCL	F	90	Lower leg	Nil	18 mo	–	Radiotherapy
31	DLCL	F	77	Lower leg	× 2	30 mo	Lymph node (12 mo)	Radiotherapy (skin and LN)
32	DLCL	M	75	Lower leg	Multiple	24 mo	Bone marrow, died of disease	Radiotherapy, chlorambucil
33	DLCL	F	93	Lower leg	Nil	18 mo	–	Radiotherapy
34	DLCL	M	73	Lower leg	Multiple (multifocal)	42 mo	Lymph nodes, died of disease	Radiotherapy, chlorambucil, rituximab, CHOP, interferon-α
35	DLCL	F	54	Scalp	Nil	24 mo	–	Radiotherapy
36	DLCL	F	79	Scalp	Multiple	12 mo	Lymph node, died of disease	Radiotherapy, CHOP

three patients, which suggests that inactivation of the p15 and p16 genes may play a role in disease progression.

In our study, loss of expression of p15 and p16 by tumor cells was identified in 63% and 90% of cases with detectable gene abnormalities, respectively. The admixed infiltrate of reactive lymphocytes and the difficulties in identifying malignant from reactive cells, particularly in low-grade PCBCL, may explain why absent protein expression was not seen in all lymphocytes within the infiltrates. Alternatively, gene inactivation may be restricted to a subpopulation of tumor cells.

In cases with no detectable gene abnormalities, normal expression of p15 and p16 by tumor cells was identified in the majority of cases studied. Reduced expression of either p15 (one case) or p16 (three cases) by tumor cells, however, may be due to either failure to identify genetic inactivation by the molecular techniques employed (false negatives) or failure of the immunohistochemical technique, although this is unlikely as positive nuclear staining of basal keratinocytes within the biopsy specimen acted as a positive internal control.

Epigenetic phenomena such as aberrant methylation of genes involved in tumor suppression, cell cycle regulation, apoptosis, DNA repair, and metastatic potential have now been identified in many malignancies. Each human cancer has a unique profile of promotor hypermethylation in which some genetic changes are shared and others are cancer type specific. These findings will help to outline the disruption of critical pathways in tumorigenesis and to derive sensitive molecular detection strategies for different tumors. Promotor hypermethylation of p73 (a tumor suppressor gene), MGMT (a DNA repair gene), and DAPK (a gene related to metastasis and invasion) have been discovered at high frequencies in systemic lymphoma (Estella *et al*, 2001). Analysis of the promotor regions of these genes in PCBCL may help to identify cases with a poor prognosis.

In summary, we have identified frequent inactivation of p15 and p16 genes in PCBCL, most commonly as a result of promotor hypermethylation. Inactivation of p16 is more common and occurs at similar frequencies in both low- and high-grade disease. Identification of p15/p16 gene abnormalities in recurrent disease suggests a role in disease progression. Investigation of other candidate genes is required.

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Table II Correlation of molecular findings with immunohistochemical evidence of p16 and p15 expression in 40 cases of cutaneous B cell lymphoma

Case number	Disease type	p15 hypermethylation	p16 hypermethylation	LOH	p15 expression	p16 expression
1 (Bx 1)	FCCL	–	–	ND	+++	+++
(Bx 2)		–	+	NL	+++	+
2	FCCL	–	–	NL	+++	+++
3	FCCL	+	+	NL	+++	++
4	FCCL	–	+	NL	ND	ND
5	FCCL	–	–	NL	+++	+++
6	FCCL	–	–	NL	+++	+++
7	FCCL	–	–	NL	+++	+++
8	FCCL	–	–	NL	TF	+++
9	MZL	–	+	NL	+++	++
10	MZL	+	–	NL	+	+++
11	MZL	+	+	NL	++	++
12	MZL	–	–	NL	+++	+++
13	MZL	+	+	NL	++	+
14	MZL	–	–	NL	++	+++
15	MZL	–	–	NL	+++	+++
16	MZL	–	–	NL	+++	++
17	MZL	+	–	NL	+	+++
18	MZL	+	–	NL	+++	+++
19	MZL	+	–	NL	+++	+++
20 (Bx 1)	MZL	–	–	NL	ND	ND
(Bx 2)	High-gde features	–	–	IFNA	+++	+++
21	MZL	–	+	NL	ND	ND
22	MZL	–	+	NL	ND	ND
23	MZL	DNA	DNA	NL	+++	+++
24	MZL	–	+	NL	ND	ND
25	MZL	–	–	NL	+++	+++
26	MZL	–	+	NL	+++	+
27	MZL	–	–	NL	+++	+++
28	Unclassifiable	–	+	NL	+++	+++
29	DLCL	–	–	NL	+++	+
30	DLCL	+	+	NL	++	+
31	DLCL	–	+	NL	+++	+ –
32	DLCL	–	–	NL	+++	+++
33	DLCL	–	–	NL	+++	++
34 (Bx 1)	DLCL	–	–	ND	+++	+++
(Bx 2)		–	+	NL	+++	++
35	DLCL	–	–	Intra p15/16	+	+ –
36	DLCL	–	+	ND	ND	ND

ND, not done; NL, no loss; TF, technical failure.Percentage of tumor cells with nuclear staining of p16/p15: +++, 75%–100%; ++, 50%–75%; +, 25%–50%; +–, < 25%.

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