High resolution loss of heterozygosity screening implicates *PTPRJ* as a potential tumor suppressor gene that affects susceptibility to non-Hodgkin's lymphoma

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Abstract

We employed a Hidden-Markov-Model (HMM) algorithm in Loss of Heterozygosity (LOH) analysis of high-density Single Nucleotide Polymorphism (SNP) array data from Non-Hodgkin´s lymphoma (NHL) entities, Follicular Lymphoma (FL) and Diffuse Large B-cell Lymphoma (DLBCL). This revealed a high frequency of LOH over the chromosomal region 11p11.2, containing the gene encoding the protein tyrosine phosphatase receptor type J (PTPRJ). Although, PTPRJ regulates components of key survival pathways in B-cells (i.e. BCR, MAPK and PI3K signaling), its role in B-cell development is poorly understood. LOH of *PTPRJ* has been described in several types of cancer but not in any hematological malignancy. Interestingly, FL cases with LOH exhibited down-regulation of *PTPRJ*, in contrast no significant variation of expression was shown in DLBCLs. Additionally, sequence screening in exons 5 and 13 of *PTPRJ* identified the G973A (rs2270993), T1054C (rs2270992), A1182C (rs1566734) and G2971C (rs4752904) coding SNPs (cSNPs). The A1182 allele was significantly more frequent in FLs and in NHLs with LOH. Significant over-representation of the C1054 (rs2270992) and the C2971 (rs4752904) alleles were also observed in LOH cases. A haplotype analysis also revealed a significant lower frequency of haplotype GTCG in NHL cases, but it was only detected in cases with retention. Conversely, haplotype GCAC was over-represented in cases with LOH. Altogether, these results indicate that the inactivation of PTPRJ may be a common lymphomagenic mechanism in these NHL subtypes and that haplotypes in *PTPRJ* gene may play a role in susceptibility to NHL, by affecting activation of PTPRJ in these B-cell lymphomas.

Keywords: LOH, *PTPRJ*, NHL, Hidden-Markov-model and cSNP

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INTRODUCTION

Non-Hodgkin's Lymphoma represents a heterogeneous group of lymphoid-derived hematological neoplasms (Swerdlow et al., 2008; Turner et al., 2010). Diffuse Large B-Cell Lymphoma (DLBCL) and Follicular Lymphoma (FL), two of the most common NHL subtypes, are originated from germinal centre B-cells, but differ in morphology, tumor biology and aggressiveness (Klein and Dalla-Favera 2008) . However, despite their clinical disparities, there are a number of shared molecular pathogenic mechanisms of these diseases, and approximately 20% of FLs transform to DLBCL by acquiring a diverse number of genomic alterations during malignant B-cell development (Martinez-Climent et al., 2003; Green et al., 2011). Because of the biological and genomic complexity observed among NHL subtypes, an understanding of their pathogenesis and etiology is still limited. Therefore, a better knowledge of common pathogenic mechanisms involved in NHL lymphomagenesis is biologically and clinically relevant.

Loss of heterozygosity (LOH) analysis through comparison of the heterozygosity of genetic markers, such as microsatellite and single nucleotide polymorphism (SNP) markers, in tumor DNA with reference to matched normal DNA can be used for the identification of novel tumor suppressor genes (TSGs). Genetic inactivation of TSGs is understood to be caused by copy-neutral (chromosomal duplications, genomic and epigenetic mutations) or copy-loss (hemizygous deletions) events (Beroukhim et al., 2006). The identification of TSGs in tumor tissue in the absence of a normal germ-line counterpart has been limited due to the absence of available germ-line DNA or proper statistical approaches that allow inference of germ-line heterozygous data. Nonetheless, the implementation of Hidden Markov model (HMM) or cohort heterozygosity comparison (CHC) approaches in the analysis of SNP array data from unpaired tumor DNA samples has permitted the detection of LOH regions and possible TSGs that might be implicated in the origin and development of cancer (Beroukhim et al., 2006; Green et al., 2010). Using this HMM approach, we identified common LOH regions across the genome of DLBCL and FL tumors including 11q11.2, targeting the *PTPRJ* gene.

Protein tyrosine phosphatase receptor type J (PTPRJ) is a type III receptor-like tyrosine protein phosphatase (RPTP), which contains an extracellular receptor composed by 8 fibronectin type III-like domains, a transmembrane region and a single phosphatase catalytic domain (Ostman et al., 1994). Several lines of evidence have shown that PTPRJ-induced dephosphorylation negatively regulates MAPK (ERK1/2), PLCG1, PI3K (p85), FLT3, B-cell receptor (BCR), PDGFRB and VEGFR2 signaling, acting as a tumor suppressor gene by controlling signaling pathways of cell growth, proliferation and angiogenesis (Kovalenko et al., 2000; Baker et al., 2001; Lampugnani et al., 2003; Tsuboi et al., 2008; Zhu et al., 2008; Sacco et al., 2009; Arora et al., 2011). Recently, Syndecan-2 (SDC2), a transmembrane heparan sulfate proteoglycan that induces cell adhesion, and Thrombospondin-1 (THBS1), a homotrimeric glycoprotein that inhibits cell growth and angiogenesis, were revealed as natural ligands of PTPRJ (Whiteford et al., 2011; Takahashi et al., 2012). A recent study has also found that PTPRJ is negatively regulated by the oncogenic effect of microRNA-328 expression in cervix and breast adenocarcinomas cell lines (Paduano et al., 2012a).

Loss of heterozygosity of *PTPRJ* has been previously reported in breast, lung, colorectal, thyroid and meningioma cancers, and implicated in the oncogenesis of these tumors (Ruivenkamp et al., 2002; Ruivenkamp et al., 2003; Iuliano et al., 2004; Petermann et al., 2011). Genetic inactivation of *PTPRJ* has not been described in any hematological malignancy, but this TSG has been described as a susceptibility gene in chronic lymphoid leukemia (CLL) and recently in childhood acute lymphoblastic leukemia (ALL) cases with *ETV6-RUNX1* genic rearrangements, indicating an important role of this RPTP in the biology of the lymphocyte and its malignant transformation (Sellick et al., 2007; Ellinghaus et al., 2012). Other protein tyrosine phosphatases (PTPs), such as PTPRC (CD45) and PTPRO, have been implicated in the lymphocyte biology by their crucial role in the regulation of key survival B-cell BCR-mediated signals through PTP-mediated dephosphorylation of SFKs and SYKs (Zhu et al., 2008; Juszczynski et al., 2009).

Although, studies on murine models and human cell lines have shown a possible regulatory role of PTPRJ throughout lymphocyte maturation and immunoresponse, its role in the B-cell development is still unknown (Tangye et al., 1998b; Lin et al., 2004). PTPRJ is moderately expressed in B-cells and upregulated once T-cells are activated. In T-cells, PTPRJ inhibits the TCR-mediated T-cell activation by dephosphorylation of LAT and PLCG1 pathways (Tangye et al., 1998b; Baker et al., 2001). A recent study determined that PTPRJ is commonly expressed in the T, NKT, NK, B, immature DC, macrophage, mast, and neutrophil cells; however, PTPRJ showed a higher expression in B-cells than in macrophages (Arimura and Yagi 2010). Conversely, in mature B-cell malignancies, a lower expression of PTPRJ has been shown in DLBCLs and FLs, in contrast to its higher expression in Mantle-cell lymphomas (MCLs) (Dong et al., 2002; Miguet et al., 2009).

Coding SNPs (cSNPs) in *PTPRJ* have been identified in colorectal and thyroid tumors as well as preferential allelic loss in patients with LOH (Ruivenkamp et al., 2002; Iuliano et al., 2004). Susceptibility studies with *PTPRJ*-mapping SNPs have associated variants with increased cancer-risk in lung squamous cell, colorectal and papillary thyroid carcinomas (Iuliano et al., 2010; Mita et al., 2010). Furthermore, Lesueur et al., (2005) found association between a *PTPRJ* haplotype and cancer-protection in breast cancer but the same haplotype did not have any effect in colorectal cancer susceptibility (Lesueur et al., 2005; Toland et al., 2008).

In the present study, using a high resolution LOH approach we identified that loss of heterozygosity of *PTPRJ* is a common event across NHL cases. Moreover, we also detected a lower transcript abundance of *PTPRJ* in FL cases with LOH than those with retention (RET) of *PTPRJ*. Additionally, the following *PTPRJ*-targeting cSNPs were identified in our population: G973A (rs2270993), T1054C (rs2270992), A1182C (rs1566734) and G2971C (rs4752904). However, we also observed a higher frequency of the A1182 (Gln276) allele in FLs, an over-representation of C1054, A1182 (Gln276) and C2971 (Asp872) alleles in NHL cases with LOH, and a high frequency of haplotype GCAC in cases with LOH. These results highlight the use of high-resolution LOH approaches in the identification of TSGs that may have an impact in normal and malignant B-cell development and the study of cSNPs that may modulate the anti-tumor functions of PTPRJ in NHL.

MATERIALS AND METHODS

Patients and control

Fresh-frozen tumor biopsies from 20 Diffuse Large B-cell Lymphoma and 24 Follicular Lymphoma patients and formalin fixed paraffin embedded tissue (FFPE) samples from 48 DLBCL cases were obtained as previously described (Green et al., 2011; Keane et al., 2011). This research was approved by the human research ethic committees of each of the participating sites. Peripheral blood samples and normal hyperplastic lymphoid tissue (HLT) were obtained from Australian Leukemia and Lymphoma Group (ALLG) Tissue Bank (Princess Alexandra Hospital, Queensland, Australia), Gold Coast Hospital (Queensland Health, Australia) and Genomics Research Centre Clinic (Griffith University, Queensland, Australia). Genomic DNA and total RNA were isolated as previously described (Green et al., 2010; Green et al., 2011; Keane et al., 2011).

High Resolution LOH analysis

Genomic DNA from 21 DLBCL and 21 FL cases was analyzed using Affymetrix 250 K Sty SNP microarrays, as previously described (Green et al., 2011). Raw data is publicly available through the gene expression omnibus (GEO), accession no. GSE22082. SNP genotyping and HMM-based LOH analysis were performed using GeneChip Genotyping Software (GTYPE; Affymetrix), following methodology previously described for inferring LOH of unpaired LOH tumor samples (Beroukhim et al., 2006; Green et al., 2010). dChipSNP and Integrative Genomics Viewer (IGV 1.5) programs were utilized to visualize the inferred LOH regions and select genes that were targeted in LOH regions with a frequency of LOH calls higher than 25% (Robinson et al., 2011). False Discovery Rates (FDR) Q-values from Genomic Identification of Significant Targets in Cancer (GISTIC) analysis of significant DNA copy number alterations were used to determine the probability of copy-loss (deletions) events in the evaluated regions (Green et al., 2011).

In order to select candidate genes for further analysis and to avoid the false positive calls intrinsic to this unpaired HMM-based LOH approach (Beroukhim et al., 2006; Heinrichs et al., 2010), we performed a microsatellite validation of five LOH regions (data not shown). The selection of candidate genes within those regions was based on their significance in lymphocyte biology. A pair of microsatellites was chosen per candidate gene, based on their proximity to the candidate gene. Herein, we only show the microsatellite-based validation for *PTPRJ*.

Microsatellite-based validation analysis

Validation of *PTPRJ* HMM-based LOH results was carried out by fragment analysis of microsatellites targeting *PTPRJ* (*D11S1350* and *D11S4183*). DNA samples from 115 controls (24 Normal hyperplastic lymphoid tissue and 91 peripheral blood samples) and 92 NHL patients (68 DLBCL and 24 FL) were evaluated. Oligonucleotides sequences were taken from uniSTS database of NCBI. All PCR reactions were optimized in a final volume of 20 µL containing 50 ng of genomic DNA, 1X Colourless GoTaq® Flexi Buffer (Promega), 2 mM of $MgCl_2$, 400 µM of dNTPs, 500 nM of each FAM-labeled primer and 0.05 units of GoTaq^{TM} DNA Polymerase (Promega). DNA was amplified using temperature cycles consisting of an initial denaturation step of 95^{o}C for 3 mins, followed by 40 cycles of 95^{o}C for 30

secs, 55-62^{o}C for 30 secs, and final step of 72^{o}C for 30 secs. Fragment analysis of alleles and genotypes for each microsatellite were determined by GeneScan® 3130 (Applied Biosystems, Austin, TX, USA), following manufacturer´s guidelines.

Hardy-Weinberg Equilibrium (HWE) was confirmed in the control population using GenePop software (version 4.0.10). A 1-sided Pearson's Chi-square test with correction for continuity was carried out to compare heterozygosity frequencies of *D11S1350* and *D11S4183* microsatellites between controls and NHL cases. The significance level was set at $\alpha < 0.05$.

PTPRJ expression analysis

Previously published normalized mean fluorescence intensity values for *PTPRJ*, from an Illumina Sentrix Human-6 (v2.0) Expression Beadchip, from cases with a known LOH status (14 FLs and 17 DLBCLs), were used to determine the effect of LOH on *PTPRJ* expression in our cohort. Normalization of this microarray data was performed as previously described by Green et al., (2011). Cell-of-Origin (COO) sub-classification of DLBCL samples was inferred from whole gene expression data of 21 DLBCL cases (Green et al., 2011), following the criteria established by Shipp et al., (2002). Based on this analysis, 10 out 21 DLBCL cases were classified as GCB-like subtype and 11 out of 21 as non-GCB-like subtype.

Validation of *PTPRJ* transcript abundance in cases with LOH and RET calls was carried out using quantitative PCR (qPCR). Total RNA from tonsils samples of 6 healthy individuals and from 41 NHL tumor tissues (20 FLs and 21 DLBCLs) with HMM-based LOH data, were reverse transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen) (Green et al., 2011)). Transcript abundance of *PTPRJ* in controls and NHL cases was determined by relative quantification ($\Delta\Delta$Ct method) of *PTPRJ* expression normalized to *RPL13A*. The set of primers targeting PTPRJ specifically amplify the isoform 1 transcript, as only this isoform contains the region encoding the catalytic domain. Sequences of oligonucleotides were: *PTPRJ-F* (5'-CAGACCCATTCAACGGATGAC-3'), *PTPRJ-R* (5'-GTGTTCGGTAAAGGTCCTTGTGT-3'), *RPL13A-F* (5'-ATCTTGTGAGTGGGGCATCT-3') and *RPL13A-R* (5'-CCCTGTGTACAACAGCAAGC-3'). Standard qPCR reactions were carried out in triplicate, in a final volume of 10µL containing 20 ng of input RNA, 200 nM of each oligonucleotide and 1X GoTaq® qPCR Master Mix (Promega). Cycling conditions and fluorescence detection was performed on a ABI 7900HT Real-Time PCR system (Applied Biosystems, Austin, TX, USA) with an initial denaturation of 95ºC for 3 min, followed by 40 cycles of 95ºC for 15 secs, and 60ºC for 1 min. Expression levels of *PTPRJ*, from genome-wide profiling and qPCR data, were compared between NHL cases with LOH and RET calls using an independent t-test ($\alpha < 0.05$).

Sequence analysis

Exons 5 and 13 of *PTPRJ* were sequenced in gDNA samples from 44 controls, 24 FL and 23 DLBCL cases. Sequences of oligonucleotides were: Exon 5-F (5'-GAAGGTGACTGCATATATCT-3'), exon 5-R (5'-AGAACATTTAGTTACTGAAAG-3'), exon 13-F (5'-CTGCCATCACTTTCTTATGAT-3') and exon 13-R (5'-CCCAAAGAGTAAGAACCAGA-3'). Bidirectional direct sequencing of 40 ng of cleaned PCR amplicon was carried out using BigDye Terminator cycle sequencing

kit v3.1 in a GeneScan® 3130 (Applied Biosystems, Austin, TX, USA), following manufacturer's guidelines.

A 2-sided Pearson's Chi-square test with correction for continuity or a Fisher's exact test was used to compare allelic and genotypic frequencies of cSNPs between controls and cases, and between cases with LOH and RET calls, respectively. Additionally, 2-sided Pearson's correlation was performed between alleles and genotypes of evaluated cSNPs between cases with LOH and RET calls. Linkage disequilibrium and comparison of haplotypic frequencies were carried out using Haploview 4.2 software (Broad Institute, 2010). Prediction of effect of cSNPs on protein function was performed using Sorting Intolerant From Tolerant (SIFT version 4.0.3) (Kumar et al., 2009) and SNPs3D (Yue et al., 2006) online tools.

RESULTS

High resolution LOH analysis identifies common LOH regions across NHL entities, and implicates LOH of PTPRJ as a common event in DLBCL and FL

The implementation of the HMM algorithm for LOH analysis allowed the identification of common LOH regions across DLBCLs and FLs (Figure 1, Table 1, Table S1). Notably, regions with known single-copy loss and mutations in DLBCL, such as *TP53BP1* and *EP300*, respectively (Takeyama et al., 2008; Morin et al., 2011; Pasqualucci et al., 2011a), were identified by our LOH analysis, thus validating our HMM-based approach (Table S1). This high resolution approach identified LOH of 11q11.2 as one of the commonly affected regions across all cases. Within this region, the *PTPRJ* gene was the most attractive candidate tumor suppressor gene, due to is well established role in regulating lymphocyte signaling. LOH of *PTPRJ* was present in 38% (16/42) of all cases; 33% (7/21) of DLBCL cases and 43% (9/21) of FL cases (Figure 2).

Unpaired LOH analysis with microsatellites validates the HMM-based LOH findings

Validation of the high resolution LOH findings for *PTPRJ* was performed by a significant reduction of heterozygosity for *D11S1350* between controls and total NHL cases, controls and FLs, and controls and DLBCLs ($P = 0.002$; $P = 0.002$; $P = 0.022$, respectively) (Table 2). Conversely, non-significant differences were observed for *D11S4183* between heterozygosity of controls and NHL cases. Discordances between these microsatellites are probably due to their positioning in relation to *PTPRJ* locus, being the result from microsatellite *D11S1350* the most informative as it is located within *PTPRJ locus* (Figure 2).

PTPRJ is down-regulated in FL cases with LOH of *PTPRJ*

Using available *PTPRJ* mean fluorescence intensity values, from an Illumina Sentrix Human-6 (v2.0) Expression Beadchip, from patients with a known LOH status (31 out of 42 NHL cases (Green et al., 2011), we could not observe any significant effect of LOH on the *PTPRJ* expression between NHL cases with RET and LOH calls (67.5 vs. 54; $P = 0.145$); however a trend towards down-regulation of *PTPRJ* was observed in FL cases with LOH (Supplementary Figure 1). This down-regulation was only confirmed in FL cases (2.05 vs 1.16; $P = 0.034$) but not in DLBCL (1.88 vs. 1.84

P = 0.962) or NHL (1.96 vs. 1.48; *P* = 0.278) cases with retention or LOH calls, respectively, by assessing PTPRJ expression by qPCR in patients with available HMM-based LOH data (41 out of 42 NHL cases) (Figure 3).

Another expression analysis performed on the DLBCL samples, considering the COO subtype, did not reveal significant changes in *PTPRJ* expression in GCB-like cases (1.7 vs. 2.4; *P* = 0.606) and in non-GCB-like cases (2.0 vs. 1.0; *P* = 0.369) with retention and LOH. Nonetheless, a trend towards PTPRJ up-regulation in GCB-like cases and PTPRJ down-regulation in non-GCB-like cases can be observed in patients with LOH (Supplementary Figure 2).

Allelic frequencies of cSNPs in the exons 5 and 13 of PTPRJ differ between NHL cases with loss of heterozygosity (LOH) calls versus those with retention (RET) calls

Previous studies have associated cSNPs located in the exons 5, 6, 7 and 13 of *PTPRJ* with colorectal and thyroid carcinomas and some of these polymorphisms have been found more frequently in patients with LOH of *PTPRJ* (Ruivenkamp et al., 2002; Iuliano et al., 2004). We performed a pilot study screening exons 5, 6, 7 and 13 of *PTPRJ* in 12 cases (8 FL and 4 DLBCL). This identified only the cSNPs G973A (Glu206), T1054C (Thr233) and A1182C (Gln276Pro), located in the exon 5, and G2971C (Glu872Asp), located in exon 13 of the *PTPRJ* gene (Table S2). Thereafter, we focused our analyses to exons 5 and 13 in an extended cohort of controls (n=44), FL (n=24) and DLBCL (n=23) cases, and compared their genotypic and allelic frequencies between these cohorts. However, only the A1182 (Gln276) allele was found to be significantly more frequent in FL cases than in controls (*P* = 0.045) (Table 3; Supplementary Table 3).

Allelic distribution of the 4 cSNPs was investigated in cases with HMM-inferred LOH calls, by comparing genotypic and allelic frequencies of G973A (rs2270993), T1054C (rs2270992), A1182C (rs1566734) and G2971C (rs4752904) in DLBCL and FL cases with LOH or retention (RET) calls (Table 4; supplementary Table 4). Overall, genotypic and allelic frequencies of rs2270992, rs1566734 and rs4752904 were highly significant between cases with LOH and RET calls. For rs2270992, a significant over-representation of C1054 versus T1054 allele was detected in patients with LOH ($p<0.0001$). The A1182 (Gln276) allele was always present in patients with LOH and in most of cases with retention of heterozygous status (*P* = 0.033 and 0.023 respectively). Moreover, a highly significant over-representation of the C2971 (Asp872) allele was observed in the majority of cases with LOH ($p<0.0001$). A significant enrichment of homozygous for the wild type G973, A1182 alleles and for the variant C1054 and C2971 alleles was observed in individuals with LOH (supplementary Table 4).

Haplotype analysis of cSNPs on exon 5 and 13 of *PTPRJ* identified the presence of a cancer-protective haplotype (GTCG) and loss of haplotype GCAC in LOH cases.

Linkage disequilibrium (LD) between rs1566734 and rs1503185 was previously determined in cases with thyroid carcinoma and adenoma (Iuliano et al., 2004). To investigate the possible LD among G973A (rs2270993), T1054C (rs2270992), A1182C (rs1566734) and G2971C (rs4752904) polymorphisms, we compared the

genotypic frequencies of these four cSNPs in controls. High LD values were observed among all 4 cSNPs, the lowest D' value was observed between A1182C (rs1566734) and G2971C (rs4752904) polymorphism (D'=0.712, *P* = 0.008). Thus, this analysis provided good evidence of a single haplotype block at this locus, which contains n=7 and n=9 possible haplotypes in controls and cases respectively (Supplementary Table 5).

Comparison of haplotypic frequencies at this locus between controls and cases revealed that haplotype GTCG was significantly less common in cases than in controls (17% vs. 6%; OR=0.33, 95% CI:0.09-0.72, *P* = 0.021). In NHL cases, this haplotype was absent in patients with LOH, in comparison to a frequency of 11.2% in patients with retention (χ^2=3.869, *P* = 0.0492). Moreover, a highly significant frequency of haplotype GCAC was observed in cases with LOH than in those with RET calls (84% vs. 36%; χ^2=18.662, *P* = 1.56 x 10^{-5}) (Supplementary Table 5). This finding highlighted a significantly low frequency of the protective haplotype GTCG in NHL cases and confirms over-representation of haplotype GCAC in NHL patients with LOH and a preferential retention of haplotype GTCG in NHL cases.

DISCUSSION

The study of common genetic alterations across FL and DLBCL cases has revealed common pathogenic mechanisms that can underlie the malignant phenotype of these B-cell lymphomas and may open a new alternative in the discovery of more effective chemotherapeutic targets (Green et al., 2011). In the present study, the implementation of HMM-based LOH analysis on high-density SNP array data from unpaired DLBCL and FL cases allowed the identification of common regions with LOH across these NHL subtypes, suggesting that the inactivation of TSGs within these regions might be a common molecular mechanism in the lymphomagenesis and aggressiveness of DLBCL and FL, as well as in the transformation of FL to DLBCL.

One of the common and most interesting LOH regions in NHL cases was mapped to the 11p11.2 locus, with *PTPRJ* identified as a potential TSG that could be inactivated in NHL lymphomagenesis. This is suggested by the significant down-regulatory effect of LOH of *PTPRJ* on its expression of *PTPRJ* in FL cases with LOH. However, it is still unclear whether events driving LOH in DLBCL cases may or may not induce an effect on *PTPRJ* expression in cases with GCB-like and non-GCB-like subtypes, due to the high heterogeneity of DLBCLs (Rosenwald et al., 2002; Pasqualucci et al., 2011b). LOH of *PTPRJ* has been previously reported in breast, lung, colorectal, thyroid and meningioma cancers, but has not been described in any hematological malignancy and its effect on expression has not been determined (Ruivenkamp et al., 2002; Ruivenkamp et al., 2003; Iuliano et al., 2004; Petermann et al., 2011). However, it is important to highlight that two SNP-based predisposition studies have previously implicated *PTPRJ* in susceptibility to CLL and to ALL cases with the genic rearrangement *ETV6-RUNX1*. (Sellick et al., 2007; Ellinghaus et al., 2012). In the present study, LOH of *PTPRJ* was identified in 4 out 10 CLL cases (unpublished data). On the other hand, the association of *PTPRJ* to *ETV6-RUNX1* genic rearrangements in ALL cases suggests that correlation analysis of LOH of *PTPRJ* with translocations occurring (i.e t(14;18), t(8;14), t(3;14) and others) in FLs and

DLBCLs is highly relevant, as these NHL subtypes have a high frequency of translocations (Rosenwald et al., 2002; Martinez-Climent et al., 2003; Morin et al., 2011; Pasqualucci et al., 2011a). Altogether this evidence supports the role of this TSG in the etiology and malignant transformation of lymphoid-origin neoplasms.

The effect that LOH of *PTPRJ* may have in B-cell derived neoplasms requires further investigation, as the role of *PTPRJ* in B-cell development has not been clearly determined. In normal B-cell development, PTPRJ (CD148) expression has been proposed as an antigen marker to differentiate human memory B-cells (PTPRJ+) from naïve B-cells (PTPRJ-) (Tangye et al., 1998a). However, in B-cell-derived neoplasms, PTPRJ expression was found to be heterogeneous across NHL subtypes, failing to classify lymphoid tissue with either memory B-cell or naïve B-cell origins. Furthermore, DLBCL and FL showed a lower and more diverse expression of PTPRJ in comparison with other NHL subtypes (MCL, CLL, BL, MALT and plasmacytoma) (Dong et al., 2002). In another study, PTPRJ expression has been proposed as a useful diagnostic marker for mantle cell lymphomas (MCL), based on its higher expression in MCLs than in CLLs and in SLLs (Miguet et al., 2009).

Although functional studies are required to determine whether LOH-driving events induce inactivation of *PTPRJ* in NHL, several lines of evidence support the hypothesis that LOH of *PTPRJ* might act as an oncogenic hit in the lymphomagenesis or progression of FLs and DLBCLs. This is based on the fact that this receptor-like protein tyrosine phosphatase (RPTP) negatively regulates key signaling for the survival and development of B-cells, such as: MAPK (ERK1/2) PLCG1, PI3K (p85), and B-cell receptor (BCR) signaling (Baker et al., 2001; Tsuboi et al., 2008; Zhu et al., 2008; Sacco et al., 2009). Interestingly, MAPK pathway has been recently identified as a common target of genetic alterations (amplification and deletions) across NHL entities (DLBCL, FL and CLL), suggesting that its deregulation might be critical for pathogenesis of these NHL lymphomas (Green et al., 2011). The significant *PTPRJ* down-regulation observed in FL cases with LOH also suggests that inactivation of *PTPRJ* might enhance oncogenic activity of MAPK signaling as PTPRJ mediates the direct dephosphorylation and inactivation of pro-proliferation ERK1/2 kinases (Sacco et al., 2009). Other lines of evidence show that PTPRJ also regulates the PI3K signaling pathway by direct dephosphorylation of p85, which is phosphorylated by SCR kinase and is simultaneously activated by PTPRJ to induce immunoreceptor signaling in B-cells and transition from pro-B to pre-B-cell (Saijo et al., 2003; Tsuboi et al., 2008; Zhu et al., 2008). Furthermore, an overlap of the roles of *PTPRJ* (CD148) and *PTPRC* (CD45) in B-cells and macrophages has been observed in knock-out (KO) experiments. In double KO mice, a severe myeloproliferative disease and a partial block of B-cell development with impairment of B-cell receptor and immunoreceptor signaling was caused by failure in PTPRJ-mediated and PTPRC-mediated dephosphorylation of the inhibitory phophotyrosine residue of SRC kinase (Zhu et al., 2008). Hence, it can be hypothesized that the effect of LOH of *PTPRJ* in NHL cases would be less deleterious than in cases with loss of both *PTPRJ* and *PTPRC*, due to their overlapping function. However, LOH of *PTPRC* was not observed in our cohort of NHL samples (data not shown). This finding instead suggests that *PTPRJ* might act as a low-penetrance gene in DLBCLs and FLs, similar to previous findings in thyroid and colorectal cancers (Ruivenkamp et al., 2002; Iuliano et al., 2004).

The molecular mechanisms that underlie the LOH-induced inactivation of *PTPRJ* in tumors are still unclear and require further investigation. The very low frequency of deletions in the *PTPRJ* locus across NHL cases and the observed reduction in the expression of *PTPRJ* in FL cases with LOH, suggest that, in our cohort of NHLs, LOH is driven by copy-neutral events, which might alter transcriptional or translational regulatory mechanisms of *PTPRJ*. One of these mechanisms could be the microduplication of the genomic region between exon 1 and 11, upstream of *PTPRJ,* as it has been shown to induce epigenetic silencing of the normal *PTPRJ* CpG island in patients with early-onset familial colorectal cancer; however, its effect on expression has not been studied (Venkatachalam et al., 2010). Moreover, it is has been revealed that *PTPRJ* expression could be also impaired by a translation attenuation mechanism, caused by the presence of alternative short upstream ORFs (uORFs) at the 5´end of *PTPRJ* (Karagyozov et al., 2008). It is possible that LOH-driving events use this mechanism to induce under-expression of PTPRJ, as low expression of PTPRJ was detected in meningioma cases with LOH (Petermann et al., 2011) and in a DLBCL cell line with LOH (unpublished data). Although somatic mutations are known as LOH-driving events, Ruivenkamp et al., (2002) failed to identify mutations after direct sequencing of the 25 exons of *PTPRJ* in NHL cases with and without LOH, indicating that mutations are not a driving cause of LOH of *PTPRJ*. This finding is supported by the low frequency of mutations observed in other types of cancer and the absence of mutations in recent exome sequencing of NHL tumors. (Forbes et al., 2011; Morin et al., 2011; Pasqualucci et al., 2011b). Nonetheless, mutations might have a dominant negative effect on the anti-tumor activity of this RPTP, as cell lines with a single copy of a deleterious mutation or deletion in the *PTPRJ* catalytic domain failed to respond to the PTPRJ-targeting antibody, Ab1, and were unable to restore the PTPRJ-associated dephosphorylation of downstream pathways (ERK1/2 and MET kinases) (Takahashi et al., 2006). Further mutational screening, epigenetic, and functional studies will be required to determine the driving cause of LOH in NHLs.

Previous sequence screenings have associated cSNPs located in exons 5, 6, 7 and 13 of the *PTPRJ* gene with colorectal and thyroid carcinomas (Ruivenkamp et al., 2002; Iuliano et al., 2004). In this study, cSNPs located within exons 5 and 13 of *PTPRJ* were identified in NHL cases and the Gln276 allele (A1182) was the only allele to be significantly more common in FL cases and controls. Nonetheless, a highly significant over-representation of the rare C1054 allele, the potentially protective Gln276 allele (A1182), and the potentially cancer-risk Asp872 allele (C2971) were observed in cases with LOH. In thyroid carcinomas, combination of homozygous for Gln276Pro, Arg326Gln and Glu872Asp polymorphisms were significantly more frequent in patients than in healthy individuals but not for each polymorphism (Iuliano et al., 2004). Haplotypes from a single haplotype block of *PTPRJ* were also identified by the allelic combination of the polymorphisms G973A (rs2270993), T1054C (rs2270992), A1182C (rs1566734), and G2971C (rs4752904) in controls. Haplotype GTCG was found to confer a protective effect for DLBCL and FL lymphomagenesis as it was more frequent in controls than in cases. The fact this haplotype was only found in patients with retention calls confirms this effect. In addition, a significant over-representation of the haplotype GCAC was observed in cases with LOH of *PTPRJ*, which both validates the analysis performed individually for each cSNP and indicates that this haplotype may have a higher effect on the function of *PTPRJ* than each polymorphism separately.

The effect of these cSNPs, individually or in a haplotype block, on the oligomerization and function of *PTPRJ*, and their relationship with LOH status in FL and DLBCL lymphomagenesis requires further study. However, it is noteworthy to mention that all analyzed cSNPs are located in encoding regions of Fibronectin type III domains, which are part of the ectodomain of PTPRJ. Particularly, the Gln276Pro (A1182C) polymorphism is a non-conservative substitution located in the exon 5, which partially encodes the second Fibronectin type III (FNIII) domain of PTPRJ, and which induces a torsional stress that potentially could affect the conformation of ectodomain receptor (Ruivenkamp et al., 2002). Conversely, the presence of the Gln276Pro polymorphism was predicted to have a no deleterious effect on the PTPRJ function. Previous association studies have linked Gln276Pro polymorphism with cancer. In this matter, the Gln276 allele failed to confer protection or susceptibility to colorectal cancer; however, significant association was observed between Gln276Pro and/or Arg326Gln alleles with susceptibility to lung squamous cell carcinoma and colorectal cancer (Mita et al., 2010)

Interestingly, the A1182 allele (Gln276) was over-represented in FL and DLBCL cases with LOH, which was also found in colorectal patients with LOH (Ruivenkamp et al., 2002). The retention of the C1182 (Pro276) allele in patients without LOH is intriguing as this variant may have an adverse effect on oligomerization and activation of PTPRJ, and consequently, may cause a constitutive activation of PTPRJ-regulated oncogenic pathways (MAPK, PI3K, PLCG1 and B-cell receptor). This hypothesis was also suggested for an *in vivo* study, as the Gln276Pro polymorphism is located in the encoding region of the antibody-targeted epitope. In this study, the antibody-mediated activation of PTPRJ inhibited angiogenesis and endothelial cell growth (Takahashi et al., 2006). The presence of this polymorphism in this epitope suggests that those cases with retention of Pro276 allele might have an adverse effect on the cellular response to this antibody. Furthermore, the high frequency observed of the wild-type Gln276 allele in FLs correlates to its indolent clinical course and suggests that the PTPRJ response to either its endogenous ligand or Ab1-like antibodies would be more sensitive than in those harboring the Pro276 allele.

Another interesting cSNP in this study was the G2971C (Glu872Asp) polymorphism. This polymorphism is a conservative substitution located in the encoding region of the 8th FNIII domain, which was predicted to cause a deleterious effect on the protein structure stability. Despite allelic and genotypic frequencies of G2971C (Glu872Asp) were not significant between controls and NHL cases, a highly significant over-representation of C2971 (Asp872) allele was observed in cases with LOH. Conversely, significant allelic retention of Asp872 allelic was found in thyroid carcinomas (Iuliano et al., 2004). Based on this, it may be possible that this cSNP might be associated with lymphomagenesis of DLBCL and FL. In another study, the Asp872 allele has been found to confer high risk to papillary thyroid carcinoma (Iuliano et al., 2010). Additionally, allele-specific epigenetic silencing of the potentially protective Glu872 allele caused by transcriptional read-through due to a microduplication upstream of *PTPRJ* promoter was observed in a patient with early-onset familial colorectal cancer (Venkatachalam et al., 2010). Hence, this finding suggests that the Asp872 allele might be preferentially expressed in cases with this microduplication and in cases with LOH.

Recent studies have also provided evidence that support the implication of *PTPRJ* in lymphomagenesis. Syndecan-2 (SDC2) and Thrombospondin-1 (THBS1) have been recently described as PTPRJ ligands, but its role in the lymphomagenesis of FL and DLBCL is still unclear (Whiteford et al., 2011; Takahashi et al., 2012). Nonetheless, it is noteworthy to mention that NHL cases who express THBS1 tend to exhibit shorter survival rates than those who do not express it (Paydas et al., 2008; Paydas et al., 2009). Hence, further studies focused on studying the role of PTPRJ and its ligands (i.e. SDSC2 and THBS1) in the development of the lymphocyte as well as in the lymphomagenesis of FL and DLBCL will be highly relevant.

Additionally, based on the tumor suppressor activities of PTPRJ in controlling pathways responsible for cell proliferation, apoptosis, angiogenesis and cell adherence, we conclude that PTPRJ is a promissory target for novel and more effective anti-cancer therapies (Takahashi et al., 2006; Paduano et al., 2012b). Interestingly, PTPRJ and anti-CD20 (Rituximab), the standard anti-NHL treatment, regulate PI3K and MAPK signaling at different targets (Vega et al., 2004; Suzuki et al., 2007; Tsuboi et al., 2008; Sacco et al., 2009). Therefore, it is important to study the genetics mechanisms that govern the expression or might disrupt the function of PTPRJ in NHL.

In summary, high-resolution LOH approaches have not only successfully identified LOH of *PTPRJ* as a common TSG-inactivating mechanism in NHL but have also implicated *PTPRJ* in the etiology and lymphomagenesis of NHL. This finding is confirmed by down-regulation of *PTPRJ* in FL cases with LOH, over-representation of cSNPs and haplotypes in patients with LOH. It is hypothesized that retention of these cSNPs could alter the ligand-mediated activation of PTPRJ and its subsequent downstream signaling; therefore, this could also affect the response of this protein tyrosine receptor to potential PTPRJ-specific immunotherapies that target epitopes harbouring cSNPs.

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Figure legends

Figure 1. High resolution (HMM) whole genome LOH analysis on Affymetrix 250 K Sty SNP microarray data from unpaired 42 NHL tumor samples inferred common LOH regions across NHL cases (Upper panel). Loss of heterozygosity of *PTPRJ* and implicated this TSG as common target of lymphomagenic hits in NHL (gray-highlighted region). In the present study, the loss of heterozygosity of TP53BP1 gene was used as a sanity-check marker. Copy-loss events in LOH regions were examined by plotting GISTIC FDR scores for deletions derived from Green et al., (2011) (Lower panel). Regions with a frequency higher than 25% were considered as common LOH regions. Deletions were identified by a GISTIC score higher than 0.01. Information about common LOH regions is detailed in Supplementary Table 1.

Figure 2. High resolution LOH analysis across chromosome 11 inferred LOH regions. A) Loss of Heterozygosity of *PTPRJ* gene is a common event (Upper panel) and is not caused by copy-loss events in NHL cases (lower panel). B) Chromosomal, genetic and transcript mapping of *PTPRJ* gene indicating location of microsatellites (*D11S1350* and *D11S4183*) that were used to validate LOH in *PTPRJ* locus.

Figure 3. Down-regulation of *PTPRJ* was observed in FL cases with LOH, suggesting that LOH in this NHL subtype is caused by LOH expression-affecting mechanisms (i.e. epigenetic silencing). Transcript abundance of *PTPRJ* was assessed by qPCR in A) NHL (n=41), B) FL (n=20) and C) DLBCL (n=21) cases with loss of heterozygosity (LOH) or retention (RET) of *PTPRJ*. A significant 1-fold decrease of *PTPRJ* expression was observed in FL cases with LOH (2.05 vs 1.16; $P = 0.034$). Conversely, no significant differences in *PTPRJ* expression were found within NHL cases (1.96 vs. 1.48; $P = 0.278$) and within DLBCL cases (1.88 vs. 1.84 $P = 0.962$) with RET and LOH calls.