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Genomic profile in anti-MAG neuropathies

Jean-Sébastien Allain

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Jean-Sébastien Allain. Genomic profile in anti-MAG neuropathies. Life Sciences [q-bio]. 2016. dumas-01755824

HAL Id: dumas-01755824

<https://dumas.ccsd.cnrs.fr/dumas-01755824v1>

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N° d'ordre :

ANNÉE 2016



THÈSE D'EXERCICE / UNIVERSITÉ DE RENNES 1
sous le sceau de l'Université Bretagne Loire

Thèse en vue du
DIPLÔME D'ÉTAT DE DOCTEUR EN MÉDECINE

présentée par

Jean-Sébastien ALLAIN

né le 26 janvier 1988 à Rennes

**Profil génomique des
neuropathies anti-MAG**

**Thèse soutenue à RENNES
le 10/10/2016**

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REMERCIEMENTS

À Monsieur le Professeur Roch Houot

Je te remercie pour ton enseignement et ton accueil chaleureux pendant mon semestre en Hématologie. J'y ai découvert l'immunothérapie sous un nouvel angle. Merci de me faire l'honneur de présider ce jury.

À Monsieur le Professeur Gilles Edan

Solliciter vos compétences et votre expérience pour juger ce travail m'a semblé une évidence. Je vous remercie pour votre encadrement lors de mon passage en Neurologie, spécialité où l'examen sémiologique prend tout son sens.

À Monsieur le Professeur Olivier Decaux

Merci pour ta bienveillance tout au long de mon internat. J'ai beaucoup appris à tes côtés. Ta profonde connaissance de la Médecine Interne et ton humanité ont beaucoup apporté à ma formation.

À Madame le Docteur Sophie De Guibert

J'ai beaucoup apprécié mon semestre à tes côtés. J'espère que mon initiation à la recherche clinique portera un jour ses fruits. Merci d'être présente pour juger mon travail.

À Madame le Docteur Morgane Pihan

Merci de m'avoir initié au monde infini des neuropathies périphériques. J'espère que les chemins de la Médecine Interne, de la Neurologie et de l'Hématologie se croiseront à nouveau.

À Monsieur le Docteur Cédric Pastoret

Ton encadrement, ta grande disponibilité, ton implication dans ce projet et ta réactivité ont été essentiels dans l'aboutissement de cet article. Ta compétence et ta rigueur m'ont énormément appris. Je te remercie pour tes encouragements, tes précieuses relectures et pour ta bonne humeur.

DEDICACES

Aux médecins, équipes et patients qui ont participé à ma formation.

À mes parents et à mes frères pour leur soutien tout au long de mes études.

À mes grands-parents.

À mon ami Pierre-Alexis.

À Nathalie, la femme de ma vie, sans qui rien n'aurait de sens.

Profil génomique des neuropathies anti-MAG

Genomic profile in anti-MAG neuropathies

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Résumé

Introduction

La neuropathie anti-MAG est une polyneuropathie acquise rare associée à une IgM monoclonale produite par un syndrome lymphoprolifératif B sous-jacent. Les études génomiques ont révélé de nombreuses mutations clonales dans les hémopathies B de bas grade.

Patients et méthodes

Les échantillons de 27 patients atteints de neuropathie anti-MAG et de 41 contrôles atteints de maladies de Waldenström (WM) ont été inclus. La mutation MYD88^{L265P} a été analysée par PCR allélique spécifique. Le statut mutationnel des gènes *MYD88*, *CXCR4*, *TP53*, *SF3B1*, *ATM* et *NOTCH1* a été étudié par un séquençage ciblé haut débit.

Résultats

Les hémopathies associées aux neuropathies anti-MAG sont 13 gammopathies monoclonales de signification indéterminée (MGUS), 12 WM, un lymphome de la zone marginale splénique (MZL) et une lymphocytose B monoclonale. Dans ce groupe, la mutation MYD88^{L265P} est présente chez 53,8% des IgM-MGUS, 91,7% des WM et chez le MZL. Nous avons identifié respectivement 3,7%, 7,4% et 7,4% de variants dans les gènes *TP53*, *ATM* et *NOTCH1*. Aucune mutation de *CXCR4* ni *SF3B1* n'a été retrouvée. Dans le groupe contrôle de WM, MYD88^{L265P} est présente dans 95,2% des cas. Des mutations de *TP53*, *ATM* et *SF3B1* sont respectivement retrouvées chez 7,3%, 7,3% et 4,9% des contrôles. Le profil mutationnel MYD88^{L265P}CXCR4^{WHIM} (19,5%) est significativement associé au groupe WM ($p < 0,05$). Les patients MYD88^{L265P}CXCR4^{WHIM} ont des taux d'hémoglobine et de plaquettes inférieurs ainsi qu'un envahissement médullaire plus important que les patients identifiés MYD88^{L265P}CXCR4^{Wild-type} ($p < 0,05$ pour chaque comparaison).

Discussion

La mutation MYD88^{L265P} est fréquemment observée dans les neuropathies anti-MAG. Le profil MYD88^{L265P}CXCR4^{Wild-type} est associé à cette neuropathie paranéoplasique définissant un nouveau lien entre le statut mutationnel et le phénotype clinique associée aux hémopathies B de bas-grade. Ces éléments ouvrent la porte à de nouvelles voies thérapeutiques.

Abstract

Background

Anti-MAG neuropathy is a rare acquired polyneuropathy associated with IgM monoclonal protein produced by an underlying B lymphoproliferative disorder. In mature small B-cell lymphoid neoplasms, genomic studies have revealed numerous clonal mutations.

Patients and Methods

Samples from 27 anti-MAG neuropathy patients and 41 Waldenström's macroglobulinemia (WM) controls were included in this study. MYD88^{L265P} was detected using allele specific PCR. Mutational status of *MYD88*, *CXCR4*, *TP53*, *SF3B1*, *ATM* and *NOTCH1* genes was assayed by targeted high throughput sequencing.

Results

Lymphoproliferative disorders associated with anti-MAG neuropathy are 13 IgM monoclonal gammopathy of undetermined significance (IgM-MGUS), 12 WM, one splenic marginal zone lymphoma (MZL) and one monoclonal B-cell lymphocytosis. In this group, MYD88^{L265P} is present in 53,8% of IgM-MGUS, 91,7% of WM and in the MZL. We identified 3,7%, 7,4% and 7,4% of variants in *TP53*, *ATM* and *NOTCH1* respectively. There are no *CXCR4* nor *SF3B1* mutations. In WM control group, MYD88^{L265P} is present in 95,2% of patients. *TP53*, *ATM* and *SF3B1* variants were found in 7,3%, 7,3% and 4,9% of controls respectively. MYD88^{L265P}CXCR4^{WHIM} mutational profile (19,5%) is significantly associated to WM group ($p < 0,05$). MYD88^{L265P}CXCR4^{WHIM} patients have lower hemoglobin, lower platelets count and higher bone marrow involvement than MYD88^{L265P}CXCR4^{Wild-type} patients ($p < 0,05$ for all comparisons).

Discussion

MYD88^{L265P} mutation is highly observed in anti-MAG neuropathy. MYD88^{L265P}CXCR4^{Wild-type} status is associated with this paraneoplastic neuropathy and defines a new impact of mutational profile on clinical phenotypes associated with small B-cell lymphoid neoplasms. It may help to define new therapeutic options.

Mots-clés: Immunoglobuline M; Polyneuropathie paranéoplasique; Gammopathie monoclonale de signification indéterminée; Maladie de Waldenström; Facteur de différenciation myéloïde-88; CXCR4

Key words: Immunoglobulin M; Paraneoplastic Polyneuropathy; Monoclonal Gammopathy of Undetermined Significance; Waldenström's macroglobulinemia; Myeloid Differentiation Factor 88; CXCR4

GLOSSAIRE

AS-PCR: Allele specific – polymerase chain reaction
ATM: Ataxia telangiectasia mutated
BM: Bone marrow
BTU: Bühlmann Titer Units
CALR: Calreticulin
CLL: Chronic lymphoid leukemia
CXCR4: C-X-C chemokine receptor type 4
DNA: Deoxyribonucleic acid
HTS: High throughput sequencing
IgM: Immunoglobulin M
IVIg: Intravenous immunoglobulins
MAG: Myelin-associated-glycoprotein
MGUS: Monoclonal gammopathy of undetermined significance
MYD88: Myeloid differentiation primary response gene 88
MBL: monoclonal B-cell lymphocytosis
MZL: Marginal zone lymphoma
NF κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells
NOTCH1: Notch homolog 1
SF3B1: Splicing factor 3B subunit 1
TP53: Tumor protein 53
VAF: Variant allele frequency
WHIM: Warts, hypogammaglobulinemia, infections, myelokathexis
WM: Waldenström's macroglobulinemia
WT: Wild type

INTRODUCTION

Demyelinating neuropathy associated with antibodies against myelin-associated-glycoprotein (MAG) is a part of the acquired chronic immune-mediated neuropathies [1]. Anti-MAG neuropathy usually affects patients after the sixth decade and is predominantly sensory in the lower limbs. Frequently, invalidating tremor, sensory ataxia and painful paresthesia are present. Evolution is usually slow, gradually causing disability but some patients experience a more severe progressive course due to ataxia and dysesthesia. Motor involvement, if present, usually occurs late in the course of the disease [2–7]. This paraproteinaemic neuropathy is related to a monoclonal B-cell which secretes a monoclonal immunoglobulin M (IgM) with an activity directed against a glycoprotein of non-compact myelin: MAG [8–10]. The underlying lymphoproliferative disorder can be a Waldenström's macroglobulinemia (WM), a non-Hodgkin's lymphoma or, more frequently, a monoclonal gammopathy of undetermined significance (MGUS) [7,11].

Whole-genome sequencing has recently improved our understanding of the molecular mechanisms of lymphoproliferative disorders. Initially described in diffuse B-cell lymphomas [12], myeloid differentiation primary response gene 88 (*MYD88*) L265P is the most prevalent somatic mutation found in WM and IgM-MGUS [13,14]. Acting as a trigger for nuclear factor- κ B (NF κ B) signaling, this mutation is an independent predictor of progression of IgM-MGUS to WM [15]. These findings suggest that *MYD88*^{L265P} mutation plays an early oncogenic role in WM pathogenesis [16]. In contrast, *MYD88*^{L265P} is absent or rarely expressed in other IgM-secreting disorders and may be used to distinguish WM from other related B-cell disorders such as IgM-secreting multiple myeloma, nodal and extranodal marginal zone lymphoma [13,16,17]. In addition to *MYD88*^{L265P}, a variety of other recurrent genetic alterations have been identified in small B cell lymphomas. At least 30 different types of somatic C-terminal mutations in *CXCR4* have been reported in WM and are similar to those observed in WHIM syndrome (warts, hypogammaglobulinemia, infections, myelokathexis), a congenital immunodeficiency disorder characterized by chronic non-cyclic neutropenia [18–21]. *CXCR4*^{WHIM} mutations block receptor internalization in myeloid cells after stimulation by its unique ligand CXCL12, resulting in persistent *CXCR4* activation [22]. *TP53* and *ATM* aberrations have also been described in WM [23]. *NOTCH1* and *SF3B1* mutations have been detected in hematologic malignancies such as chronic lymphoid leukemia (CLL) [24–26]. A better knowledge of these somatic mutations may be relevant for the management of anti-MAG neuropathy patients. Therefore, our purpose is to assess clonal mutations frequency in patients with this rare

polyneuropathy.

PATIENTS AND METHODS

Patients and samples

We evaluated 27 cases of anti-MAG neuropathy (anti-MAG group) from the clinical files of Rennes University Hospital, Saint-Brieuc General Hospital and Cesson-Sévigné Private Hospital. All patients have IgM monoclonal gammopathy, anti-MAG antibodies, clinical and electrophysiological evidence of anti-MAG neuropathy according to Peripheral Nerve Society guidelines for chronic inflammatory demyelinating polyradiculoneuropathy made by European Federation of Neurological Societies [7]. Anti-MAG antibodies were considered positive if superior to 1000 Bühlmann Titer Units (BTU) cutoff in ELISA. Nearby clinical, biological and histological data were retrospectively collected. Bone marrow (BM) infiltration was evaluated by immunohistochemistry on trephine biopsy and/or by flow cytometry on BM aspirate. BM aspirates and peripheral blood samples were all collected as part of routine care. Cells were stocked in the Hematological Laboratory from Rennes University Hospital. No new samples were collected. We compared the anti-MAG group to 41 patients with WM (WM group). WM was defined by the presence of an IgM monoclonal gammopathy of any concentration associated with medullar involvement by lymphoplasmocytic lymphoma according to consensus criteria diagnosis of the international recommendations [27–29]. All patients provided informed consent. Their participation was approved by the institutional ethics committee of Rennes University Hospital (notice n° 15.84).

MYD88^{L265P} detection by allele specific - polymerase chain reaction

Genomic DNA was extracted from BM or blood nuclear cells using Qiagen DNA extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. MYD88^{L265P} (NM_002468:ex5:c.794T>C:p.L265P) was screened by two allele specific – polymerase chain reactions (AS-PCR) using different forward primers with a single base substitution and the same reverse primer: forward MYD88^{Wild-type(WT)} 5'-GTGCCCATCAGAAGCGCCT-3', forward MYD88^{L265P} 5'-GTGCCCATCAGAAGCGCCC-3', reverse 5'-AGGAGGCAGGGCAGAAGTA-3'. Briefly, 250ng of DNA was amplified to a final volume of 50µL containing 0.2µM of primers (forward L265P or WT and reverse), 2mM of MgCl₂, 0.2mM of dNTPs, 2.5U of TaqGold DNA polymerase and the Gold buffer. PCR consisted of an initial denaturation step (15 min at 94°C) followed by 35 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 10 min. Results were defined

as negative, weak positive or positive depending on visualization of PCR products by 3% agarose gel electrophoresis as shown in **Figure 1**. DNA extracted from a mutated patient was used as a positive control and polyclonal control IVS0000 as a negative control.

High throughput sequencing

Mutational status of *MYD88*, *CXCR4*, *TP53*, *SF3B1*, *ATM* and *NOTCH1* genes were determined by targeted high throughput sequencing (HTS). A total of 234 enrichment primers were undertaken by the Fluidigm design team with a target size range of 150–160 bp. Target enrichment was performed according to the manufacturer's instructions using the Access Array System for Illumina Sequencing Systems (Fluidigm San Francisco, CA, USA). Then, purified libraries were sequenced with the Illumina MiSeq using MiSeq Reagent kit v2 (MS-102-2002) 300 cycles (Illumina, San Diego, CA, USA). Data were analysed using house bioinformatics pipeline. Variants were analysed according to the depth and variant allele frequency (VAF), their position and function in the coding sequence (hg19 refGene) [30], the cancer specific database COSMIC (Catalogue of Somatic Mutations in Cancer) [31], common polymorphism database (Exome Aggregation Consortium v0.3.1) [32] and bioinformatics prediction scores PolyPhen (Polymorphism phenotyping v2) [33] and SIFT (Sorting intolerant from tolerant) [34]. Somatic mutations were defined as frameshift, stop gain or missense variants with a VAF higher than 2%, at least 5 reads, with a SIFT score <0,05, a not-“benign” PolyPhen prediction and not reported as a polymorphism.

Statistical analysis

Categorical variables were compared with the use of Fisher's exact probability test, and ordinal variables with the use of the Mann-Whitney U test. To perform calculations in serum protein, we arbitrarily chose a value of 1g/L when monoclonal protein was unquantifiable on immunoelectrophoresis. A Pearson correlation coefficient was used for linear comparisons. P values of 0,05 or less were considered to indicate statistical significance. Calculations were performed with the use of the R statistical package (R Foundation for Statistical Computing).

RESULTS

The clinical, biological and molecular characteristics of the two groups are summarized in **Table 1**.

In anti-MAG group, all patients (n=27) have symptomatic neuropathy. Paresthesia and dysesthesia are the first common manifestation of the neuropathy in 16 patients (59%). The

underlying lymphoproliferative disorders are 13 IgM-MGUS (48,2%), 12 WM (44,4%) including 9 indolent and 3 symptomatic forms, one marginal zone lymphoma (MZL) with a splenic subtype and one monoclonal B-cell lymphocytosis (MBL) with a 5 Matutes score (3,7%). Two patients were previously treated for neuropathy: one with intravenous immunoglobulins (IVIg) and one with IVIg then Cyclophamide. No subject received a treatment for hematological indication before sample collection. Sequencing was assessed in 21 BM aspirates and 6 peripheral blood samples. MYD88^{L265P} is detected in 19 subjects (70,3%). Among them, 18 (91,7%) are identified by AS-PCR and 10 (52,7%) by HTS assays. Nine discordant results are exclusively positive in AS-PCR (including 5 “weak positives” and 4 “positives”) and one in HTS assays. We identified MYD88^{L265P} in 7/13 IgM-MGUS (53,8%), in 11/12 WM (91,7%) and in the splenic MZL. We found one *TP53*, 2 *NOTCH1* and 2 *ATM* mutations which are all associated with a MYD88^{L265P} status. We did not find *CXCR4* or *SF3B1* variants. Results are presented in **Figure 2** and details in **Table 2**. Karyotype done in the MZL patient (n°2) shows trisomy 19 and an abnormality on chromosome 17 without *TP53* deletion. Patient n°15 also carries calreticulin (*CALR*) mutated essential thrombocythemia.

According to the *MYD88* status, no significant difference was found in the clinical presentation of anti-MAG neuropathy: age (p=0,68), paresthesia/dysesthesia (p=0,62), sensory deficit (p=0,63), motor deficit (p=0,65), ataxia (p=1), pain (p=0,4). Neither in the biological data: anti-MAG antibodies titer (p=0,79), hemoglobin (p=0,85), platelets count (p=0,85), white blood cells count (p=0,87), monoclonal protein (p=0,60).

In WM group, there are 29 untreated and 12 previously treated patients. All the treated patients were in relapse at the time of sampling. Among them, 8 received one line of chemotherapy, 2 two lines and 2 three lines including combinations of Rituximab, Cyclophosphamide, Dexamethasone, Bendamustine, Fludarabine, Bortezomib and Idelalisib. There was no case of familial WM. Mutational analyses were performed in 24 BM aspirates (20 at diagnosis, 4 at relapse) and 17 peripheral blood samples (9 at diagnosis, 8 at relapse). In 4 cases, BM involvement data is missing due to an absence of available archived BM biopsies. We detected 39 (95,2%) MYD88^{L265P} cases. All of which were detected with AS-PCR assays and 28 (68,3%) with HTS assays. We found a significant correlation between clonal cells infiltration in flow cytometry and MYD88^{L265P} VAF (**Figure 3**). *CXCR4*^{WHIM} is detected in 8 (19,5%) cases in the WM group. All patients with *CXCR4*^{WHIM} also harbored the MYD88^{L265P} mutation. Patients with the MYD88^{L265P}*CXCR4*^{WHIM} status have significantly lower hemoglobin, a lower platelets count and a higher BM involvement than patients with a MYD88^{L265P}*CXCR4*^{WT} profile as shown in **Table 3**. *TP53*, *ATM* and *SF3B1* variants were found in 3, 3 and 2 cases respectively.

We did not identify *NOTCH1* variants. Results are presented in **Figure 2** and in **Table 4**. More detailed information is provided in the supplementary **Table S1**.

DISCUSSION

Summary of evidence

This is the first study that assesses somatic mutations in B lymphoproliferative disorders associated with anti-MAG neuropathy. The demographic features of our patients do not differ from previous studies: median age at diagnosis of 69 years and sex ratio of 2:1 favoring males [4,35,36]. MYD88^{L265P} is expressed in 66,7% of anti-MAG neuropathy with 91,7% and 52,7% in patients with WM and with IgM-MGUS respectively. Similar proportions are already described in literature [13–17,37–39] and also seen in our WM control group (95,2%).

The principle finding from this study is the specific mutational MYD88^{L265P}CXCR4^{WT} presentation of anti-MAG neuropathy patients. Indeed, we did not observe any anti-MAG neuropathy patient with the MYD88^{L265P}CXCR4^{WHIM} profile compared to 19,5% in WM group. It has been shown that somatic MYD88 and CXCR4 mutations are determinants in the clinical presentation of WM. MYD88^{L265P}CXCR4^{WHIM/Non-sense} patients present with higher BM disease involvement, higher serum IgM levels and symptomatic disease including hyperviscosity syndrome [21]. Mutations in CXCR4 are also associated with a lower hemoglobin, white cell count and platelets count [40]. We found similar results in hemoglobin, platelets count and medullar involvement comparing WM patients according to CXCR4 status. All this suggests a higher activity disease therefore requiring more intensive treatment. By demonstrating an association between anti-MAG neuropathy and MYD88^{L265P}CXCR4^{WT} profile in clonal B cells, our study highlights a new clinical impact of MYD88 and CXCR4 expression in lymphoproliferative disorders. We hypothesize that CXCR4^{WT} patients may develop a clinical anti-MAG neuropathy over time due to a less aggressive course of their hematological malignancy.

Interestingly, we also report somatic mutations rarely described in WM. In 53 WM patients among the anti-MAG and WM groups, 5 (9,4%) *ATM*, 3 (5,7%) *TP53*, 2 (3,8%) *SF3B1* and 2 (3,8%) *NOTCH1* somatic variants are identified by our HTS assay. These somatic mutations are already well-known in CLL [24]. *ATM* plays a central role in maintaining genomic integrity and is required to prevent B-cell lymphomagenesis [41]. Somatic mutations or deletions (chromosome 11q) of *ATM* are observed in several sporadic human lymphoid malignancies [42,43]. Thus, deletions of chromosome 11q are associated with a severe clinical phenotype

and reduced survival in CLL [44]. In our WM control group, the 3 ATM variants with VAF close to 50% (p.G2023R, p.H1264Fs and p.S333F) can be considered as germ line variants heterozygosity. These germ line variants can be considered pathogenic because they may influence disease progression through loss of the other allele [45,46]. Located on the short arm of chromosome 17, *TP53* is a transcription factor. It functions as a key tumor suppressor and as a master regulator of various signaling pathways. TP53 protein plays a crucial role in a variety of cellular processes, including cell cycle arrest, apoptosis, DNA repair, genomic stability, cell differentiation, and senescence [47–50]. Its importance is reflected by the fact that it can be altered in about 50% of human cancers [51,52]. In one of our patients with anti-MAG neuropathy, *TP53* mutation (p.S241F) was also be associated with the *CALR* mutated essential thrombocythemia [53]. *SF3B1* gene encodes a component of spliceosome and is frequently mutated in 10-17% of cases of CLL [54–56]. While no links between mutations of genes for spliceosome machinery and CLL pathogenesis have yet been described, *SF3B1* mutations have added independent prognostic value [57]. The oncogenic role for NOTCH signaling has been well defined in T-cell malignancies [58,59] and more recently in B-cell malignancies [60]. Its mutational incidence has been reported with a variable range in CLL [24,25,61]. NOTCH1 protein belongs to a family of four transmembrane receptors (i.e. NOTCH1-4) and five ligands, expressed on the cell surface. It acts as a ligand-activated transcription factor, affecting the implementation of differentiation, proliferation and apoptotic programs [62]. Among others, NOTCH signaling activates antiapoptotic mechanisms via NFκB pathways [63]. As shown by Nadeu et al. in CLL [46], our findings suggest that targeted HTS may become a common approach for the assessment of genomic alterations and may be relevant for management strategies of anti-MAG neuropathy patients.

Limitations

Among lymphomas, WM presents one of the most important discordance in clonal cells infiltration between BM and peripheral blood [64,65]. This should be remembered when interpreting sequencing results regarding the sample infiltration. It also depends of the sensitivity of the used technique. Our AS-PCR reached a sensitivity of 0.1% of mutated cells which can detect *MYD88* mutations in low infiltrated samples observed by conventional immunohistochemistry of flow cytometry. In WM, Xu et al. recently showed that AS-PCR has a similar sensitivity to detect *MYD88*^{L265P} mutation in BM and in peripheral blood. Among cases with *MYD88*^{L265P} mutation detected in BM, 98% are also positive in peripheral blood. This level declines to 83% in peripheral blood assays of IgM-MGUS [66], which are defined

with no histological evidence of infiltration by lymphoplasmocytic cells [37]. This demonstrates a high risk of false negative results in low infiltrated samples (peripheral blood samples, IgM-MGUS) compared to massive clonal cells infiltration in BM samples of symptomatic WM. That is why only positive AS-PCR results can be interpreted in a low infiltrated samples to avoid false negative results. We must be careful with a test performed on a low infiltrated sample, such as blood, before reaching the conclusion of a false identification of WM with MYD88^{WT} status. Therefore in clinical practice, we propose to establish MYD88 status with medullar assessment in some patients whose findings are negative in peripheral blood. Moreover, we selected a positive threshold of 2% for HTS analysis in order to avoid false positive results. This decrease in sensitivity explains that 57/58 (98,3%) MYD88^{L265P} variants have been assessed with AS-PCR and only 37/58 (63,8%) with HTS assays among our two groups of patients. We also show a relevant correlation between cells infiltration and VAF. When no clonal infiltration is found with flow cytometer, VAF may be inferior to HTS sensitivity threshold. Consequently, detection of *CXCR4*, *TP53*, *SF3B1*, *ATM* and *NOTCH1* mutations with our routinely performed targeted HTS may not be relevant in discrepant cases (MYD88^{L265P} AS-PCR positive / HTS negative) and especially in poorly infiltrated samples. Additionally, we identify CXCR4^{WHIM} in 19,5% of cases in the WM group. In literature, CXCR4^{WHIM} is seen in 27–43% of untreated WM [20,40,67,68]. Variations in studies probably reflect differing degrees of infiltration, but also diverse techniques and primers used to assess the mutation. So, we recommend reserving HTS to phenotypically infiltrated sample.

Secondly, we did not assay anti-MAG antibodies in the WM control group in the absence of stored serum. However, no patient of the WM group presented with clinical polyneuropathy. Furthermore, only 6,7% of patients with IgM monoclonal gammopathy without neuropathy present with anti-MAG antibodies [69].

We found a relevant and statistical difference between CXCR4^{WHIM} expression in anti-MAG neuropathy and WM groups. This is probably due to a lack of power, that there is no statistical significance comparing subgroups of WM patients with neuropathy to those WM without neuropathy. Indeed, anti-MAG neuropathy is a rare polyneuropathy with an estimated prevalence of about 0,42 per 100 000 [70]. In addition, we identified only 5 patients with an IgM-MGUS diagnosis and available stored cells in our center. Consequently, we did not compare anti-MAG patients to a second control group composed of IgM-MGUS.

Finally, it should be kept in mind that we did not assess cytogenetic aberrations with karyotype except in one patient. In a prospective study of 174 untreated WM, 6q deletions (30%), trisomy

18 (15%), 13q deletions (13%), 17p deletions (8%), trisomy 4 (8%), and 11q deletions (7%) were found [23]. Therefore, we cannot exclude a misestimating of *TP53* and *ATM* alterations due to 17p and 11q deletions respectively.

Perspectives

So far, no treatment has proved to be effective in anti-MAG neuropathy and all randomized controlled trials have been negative [35,71]. MYD88 is known to play a role of adaptor in modulating Toll-like receptor and interleukin-1 receptor signaling through interactions with interleukin-1 receptor-associated kinase (IRAK). In addition, MYD88^{L265P} triggers Bruton tyrosine kinase (BTK) and downstream NFκB signaling independent of IRAK1 [13,39,72–75]. Consequently, high frequency of MYD88^{L265P} variants in anti-MAG neuropathy supports NFκB pathway targeting and might represent a promising treatment option. Moreover, ibrutinib is a molecule inhibitor of BTK that triggers apoptosis of WM cells [75,76]. In 2015, ibrutinib demonstrated a sustained efficacy in previously treated patients with WM. In this study, response to ibrutinib treatment is affected by MYD88 and CXCR4 mutation status since response rates are highest among patients with MYD88^{L265P}CXCR4^{WT} (100% overall response rate and 91.2% major response rate) [77]. In vitro experiments have also shown that engineered CXCR4^{WHIM} mutant cells show muted CXCR4 receptor internalization, which results in enhanced and prolonged AKT and ERK activation and contribute in resistance to the suppressive effects of ibrutinib and many others antineoplastic agents [67,78]. Thus, MYD88^{L265P}CXCR4^{WT} status may support therapeutic implications of ibrutinib for patients with anti-MAG neuropathy. However, we need further studies to expand on these findings and hypotheses.

CONCLUSION

As in WM and IgM-MGUS, MYD88^{L265P} mutation is highly observed in anti-MAG neuropathy. MYD88^{L265P}CXCR4^{WT} is associated with anti-MAG neuropathy and defines a new impact of mutational status on clinical phenotypes associated with small B-cell lymphoid neoplasms which may help to define new therapeutic options in the future.

ACKNOWLEDGEMENTS

We would like to thank all patients and clinicians for their participation. We thank Ghislaine Robert and Alex Robert for revising the English. This work was produced with the software HEMSYS® dedicated to hematology which allows the extraction and analysis of patient data.

FUNDING

This study was supported by authors own institution (Rennes University Hospital and Rennes 1 University).

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Table 1. Clinical, biological and mutational characteristics of anti-MAG and WM groups

Characteristics	Anti-MAG group (n=27)	WM group (n=41)	p
Male sex - n (%)	18 (66,7)	29 (70,7)	
Median age (range) - year	69 (44 - 86)	74 (49 - 86)	
Median disease duration (range) - year	3 (0 - 13)	-	
Median anti-MAG titers (range) - BTU	38 589 (3 620 - >70 000)	-	
Anti-MAG titers > 10 000 BTU - n (%)	24 (88,9)	-	
Symptoms - n (%)			
Paresthesia / Dysesthesia	21 (77,8)	-	
Sensory deficit	22 (81,5)	-	
Motor deficit	7 (25,9)	-	
Ataxia	12 (44,4)	-	
Pain	7 (25,9)	-	
Light chain isotype† - n (%)			
Kappa	23 (85,2)	34 (82,9)	NS
Lambda	5 (18,5)	7 (17,1)	
Mean monoclonal protein (± SD) - g/L	5,3 (± 6,2)	16,3 (± 13,4)	< 10 ⁻³
Mean gammaglobulin (± SD) - g/L	13,8 (± 5,3)	20,4 (± 11,4)	< 0,05
Mean hemoglobin (± SD) - g/dL	13,8 (± 1,3)	11,4 (± 2,4)	< 10 ⁻⁴
Mean platelet count (± SD) - per mm ³	273 000 (± 157 000)	222 000 (± 120 000)	< 0,05
Mean WBC count (± SD) - per mm ³	6 700 (± 2 530)	7 300 (± 3 400)	NS
Mean neutrophil count (± SD) - per mm ³	3 800 (± 1 330)	4 100 (± 2200)	NS
Mean lymphocyte count (± SD) - per mm ³	2 170 (± 2 040)	2 200 (± 2 500)	NS
Mutation - n (%)			
MYD88 ^{L265P} ‡	19 (70,4)	39 (95,2)	< 0,05
CXCR4 ^{WHIM}	0 (0)	8 (19,5)	< 0,05
TP53 variant	1 (3,7)	3 (7,3)	NS
SF3B1 variant	0 (0)	2 (4,9)	NS
ATM variant	2 (7,4)	3 (7,3)	NS
NOTCH1 variant	2 (7,4)	0 (0)	NS

Abbreviations : BTU, Bühlmann titer units; MAG, myelin associated glycoprotein; NS, not significant; SD, standard deviation; WBC, white blood cells; WHIM, warts, hypogammaglobulinemia, infections, myelokathexis ; WM, Waldenström's macroglobulinemia; WT, Wild type.

† One patient in the anti-MAG group has biclonal IgM gammopathy.

‡ MYD88 status includes results from allele specific – PCR and high throughput sequencing.

Table 2. Somatic clonal mutations in anti-MAG neuropathy

Patient number	Hemopathy	Bone marrow infiltration (%)	MYD88L265P AS-PCR status	MYD88 HTS status (VAF)	CXCR4 HTS status (VAF)	TP53 HTS status (VAF)	NOTCH1 HTS status (VAF)	SF3B1 HTS status (VAF)	ATM HTS status (VAF)
1	MBL	2	Negative	-	-	-	-	-	-
2	MZL	55	Positive	L265P (20%)	-	-	-	-	-
3	IgM-MGUS	0	Weak positive	-	-	c.722C>T;p.S241F (3%)	-	-	-
4	IgM-MGUS	0	Positive	L265P (2%)	-	-	-	-	-
5	IgM-MGUS	0	Weak positive	-	-	-	-	-	-
6	IgM-MGUS	0	Weak positive	-	-	-	-	-	-
7	IgM-MGUS	0	Weak positive	-	-	-	-	-	-
8	IgM-MGUS	0	Weak positive	-	-	-	-	-	-
9	IgM-MGUS	0	Negative	L265P (2%)	-	-	-	-	-
10	IgM-MGUS	0	Negative	-	-	-	-	-	-
11	IgM-MGUS	0	Negative	-	-	-	-	-	-
12	IgM-MGUS	0	Negative	-	-	-	-	-	-
13	IgM-MGUS	0	Negative	-	-	-	-	-	-
14	IgM-MGUS	0	Negative	-	-	-	-	-	-
15	IgM-MGUS	0	Negative	-	-	-	-	-	-
16	WM	15	Positive	L265P (5%)	-	-	c.6346T>G;p.Y2116D (3%)	-	c.8099A>G;p.K2700R (2%)
17	WM	5	Positive	L265P (3%)	-	-	c.7460A>G;p.Q2487R(5%)	-	-
18	WM	5	Positive	L265P (7%)	-	-	-	-	c.8294G>T;p.G2765V (5%)
19	WM	90	Positive	L265P (4%)	-	-	-	-	-
20	WM	70	Positive	-	-	-	-	-	-
21	WM	70	Positive	L265P (19%)	-	-	-	-	-
22	WM	25	Positive	L265P (7%)	-	-	-	-	-
23	WM	15	Positive	L265P (4%)	-	-	-	-	-
24	WM	10	Positive	-	-	-	-	-	-
25	WM	10	Positive	-	-	-	-	-	-
26	WM	5	Positive	-	-	-	-	-	-
27	WM	10	Negative	-	-	-	-	-	-

Abbreviations: AS-PCR, allele specific polymerase chain reaction; IgM-MGUS, IgM monoclonal gammopathy of undetermined significance; HTS, high throughput sequencing; MBL, monoclonal B-cell lymphocytosis; MZL, marginal zone lymphoma; VAF, variant allelic frequency; WM, Waldenström's macroglobulinemia.

Table 3. Comparison of data dependent on CXCR4 status in WM group

Characteristics - mean (\pm SD)	MYD88 ^{L265P} CXCR4 ^{WHIM} (n=8)	MYD88 ^{L265P} CXCR4 ^{WT} (n=31)	p
Age – year	72,1 (\pm 9,6)	70,2 (\pm 10,4)	NS
Monoclonal protein - g/L	25,4 (\pm 17,4)	13,8 (\pm 11,8)	NS
Hemoglobin - g/dL	9,8 (\pm 2,0)	11,9 (\pm 2,4)	< 0,05
Platelet count - per mm ³	142 000 (\pm 75 000)	247 000 (\pm 124 000)	< 0,05
WBC count - per mm ³	5 390 (\pm 2 040)	7 500 (\pm 3 500)	NS
Neutrophil count - per mm ³	2900 (\pm 1 500)	4 050 (\pm 1 720)	NS
Lymphocyte count - per mm ³	1 710 (\pm 1 000)	2 380 (\pm 2 780)	NS
Bone marrow involvement - %	69,4 (\pm 28,7)	35,6 (\pm 26,8)	< 0,05

Abbreviations : NS, not significant; SD, standard deviation; WBC, white blood cells; WHIM, warts, hypogammaglobulinemia, infections, myelokathexis ; WM, Waldenström's macroglobulinemia; WT, Wild type.

The two MYD88^{WT}/CXCR4^{WT} patients were not included in statistical analyses because of the low cases number.

Table 4. Genomic profile of Waldenström's macroglobulinemia

Number of cases	Bone marrow infiltration (%)	MYD88 ^{L265P} AS-PCR status	MYD88 HTS status (VAF)	CXCR4 HTS status (VAF)	TP53 HTS status (VAF)	NOTCH1 HTS status (VAF)	SF3B1 HTS status (VAF)	ATM HTS status (VAF)
17		Positive	L265P	-	-	-	-	-
5		Positive	L265P	p.S338X	-	-	-	-
4		Positive	-	-	-	-	-	-
4		Weak Positive	-	-	-	-	-	-
1	45	Positive	L265P (25%)	p.S338X (20%)	p.L206fs (23%)	-	-	-
1	80	Positive	L265P (23%)	p.T318fs (29%)	-	-	-	-
1	80	Positive	L265P (37%)	p.R334X (46%)	-	-	-	-
1	<i>md</i>	Positive	L265P (53%)	-	p.R259M (3%)	-	-	-
1	80	Positive	-	-	p.P177R (8%)	-	-	-
1	30	Positive	L265P (7%)	-	-	-	p.K666R (33%)	-
1	70	Positive	-	-	-	-	p.K700E ; p.R775G (19 ; 18%)	-
1	80	Positive	L265P (43%)	-	-	-	-	p.G2023R (51%)
1	40	Weak Positive	-	-	-	-	-	p.H1264fs (51%)
1	<i>md</i>	Negative	-	-	-	-	-	p.S333F (47%)
1	10	Negative	-	-	-	-	-	-

Abbreviations: AS-PCR, allele specific – polymerase chain reaction; *md*, missing data; HTS, high throughput sequencing; VAF, variant allelic frequency.

Variant allelic frequencies and nucleotide changes are detailed for each variant in supplementary **Table S1**.

List of Figures

- **Figure 1.** Agarose gel-based conventional AS-PCR assay for detection of MYD88^{L265P}

Abbreviations: WT, wild type.

- **Figure 2.** Genomic profile of anti-MAG neuropathy and Waldenström's macroglobulinemia groups

Abbreviations: MAG, myelin associated glycoprotein; MBL, monoclonal B-cell lymphocytosis; IgM-MGUS, Immunoglobulin M monoclonal gammopathy of undetermined significance; MZL, marginal zone lymphoma; WM, Waldenström's macroglobulinemia.

The columns represent individual patients coded on the base of gene status. Each patient is represented by a virtual column: black, somatically mutated; grey, somatically mutated with discordance between AS-PCR and HTS assays; white, consistent with wild-type.

CXCR4 mutation status includes all WHIM mutations.

- **Figure 3.** Correlation between clonal cells infiltration in flow cytometry and MYD88^{L265P} variant allelic frequency

Abbreviations: AS-PCR, allele specific – polymerase chain reaction; FCM, flow cytometry; VAF, variant allelic frequency

In 34 patients, bone marrow aspirations were performed. Samples were stored and used to estimate clonal cells infiltration with flow cytometry. Each symbol (circle, triangle, diamond) represents a patient according to its MYD88^{L265P} status with AS-PCR. Samples with no clonal cells infiltration are classified as IgM-MGUS.

Figure 1. Agarose gel-based conventional AS-PCR assay for detection of MYD88^{L265P}

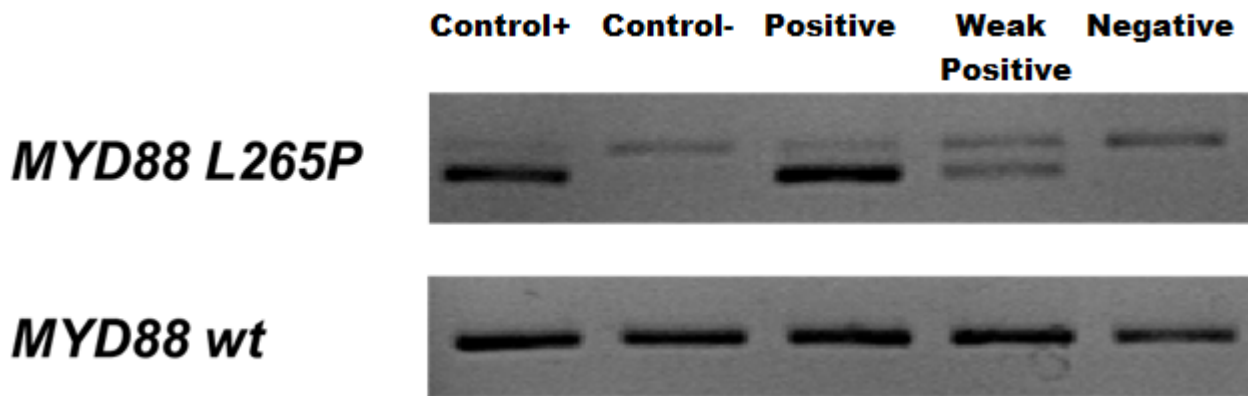
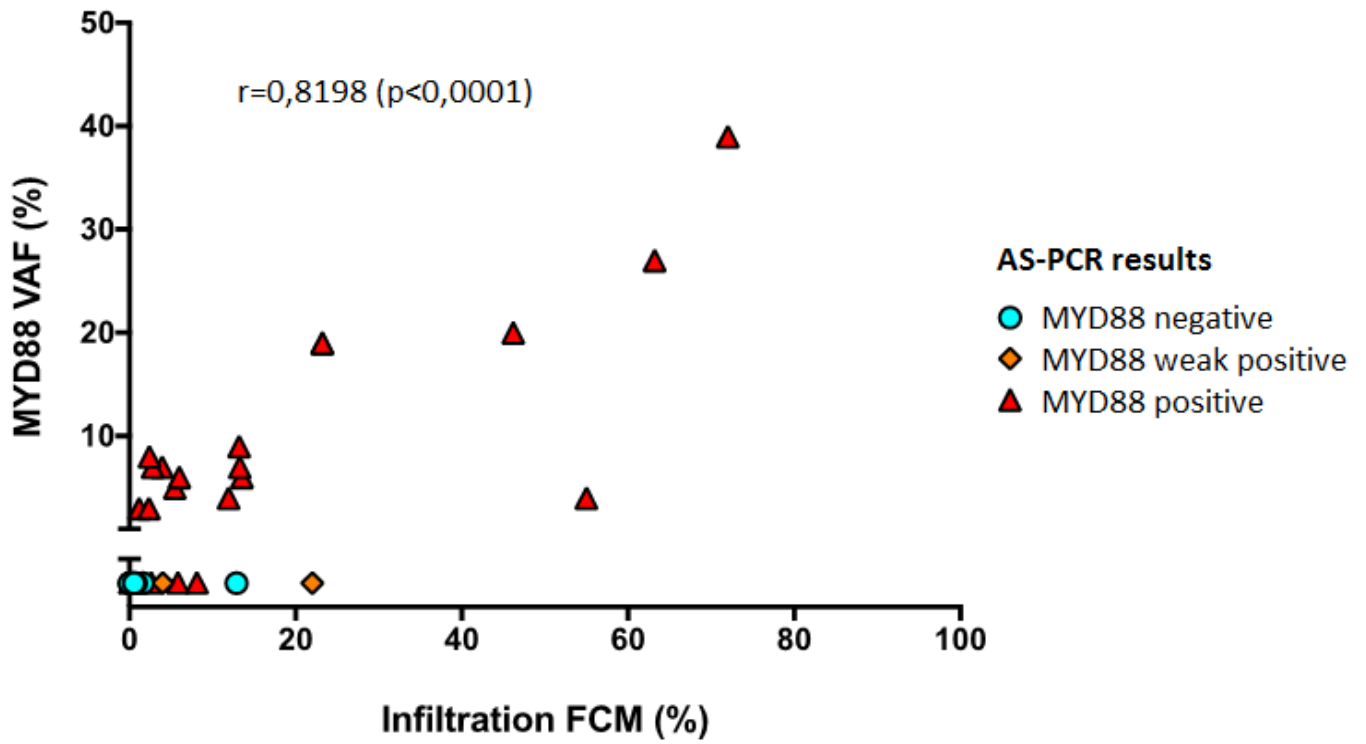


Figure 3. Correlation between clonal cells infiltration in flow cytometry and MYD88^{L265P} variant allelic frequency



Supplementary Information

- **Supplementary Table S1.** Genomic profile of Waldenström's macroglobulinemia

Supplementary Table S1. Genomic profile of Waldenström's macroglobulinemia

Patient number	Bone marrow infiltration (%)	MYD88L265P AS-PCR status	MYD88 HTS status (VAF)	CXCR4 HTS status (VAF)	TP53 HTS status (VAF)	NOTCH1 HTS status (VAF)	SF3B1 HTS status (VAF)	ATM HTS status (VAF)
1	45	Positive	L265P (25%)	c.1013C>g.p.S338X (20%)	c.616_642del:p.L206fs (23%)	-	-	-
2	80	Positive	L265P (23%)	c.953_954insA:p.T318fs (29%)	-	-	-	-
3	10	Positive	L265P (6%)	c.1013C>G:p.S338X (5%)	-	-	-	-
4	95	Positive	L265P (21%)	c.1014_1015insC:p.S338fs (21%)	-	-	-	-
5	95	Positive	L265P (6%)	c.1013C>A:p.S338X (6%)	-	-	-	-
6	80	Positive	L265P (37%)	c.1000C>T:p.R334X (46%)	-	-	-	-
7	70	Positive	L265P (39%)	c.1013C>A:p.S338X (38%)	-	-	-	-
8	80	Positive	L265P (4%)	c.1013C>A:p.S338X (4%)	-	-	-	-
9	<i>md</i>	Positive	L265P (3%)	-	c.746G>T:p.R249M (3%)	-	-	-
10	80	Positive	-	-	c.530C>G:p.P177R (8%)	-	-	-
11	30	Positive	L265P (7%)	-	-	-	c.1197A>G:p.K666R (33%) c.2098A>G:p.K700E (19%) & c.2323C>G:p.R775G (18%)	-
12	70	Positive	-	-	-	-	-	-
13	80	Positive	L265P (43%)	-	-	-	-	c.6067G>A:p.G2023R (51%)
14	40	Weak Positive	-	-	-	-	-	c.3791delA:p.H1264fs (51%)
15	50	Positive	-	-	-	-	-	-
16	50	Positive	L265P (19%)	-	-	-	-	-
17	<i>md</i>	Positive	L265P (8%)	-	-	-	-	-
18	20	Positive	L265P (6%)	-	-	-	-	-
19	25	Positive	L265P (14%)	-	-	-	-	-
20	10	Positive	-	-	-	-	-	-
21	20	Positive	L265P (14%)	-	-	-	-	-
22	5	Positive	L265P (5%)	-	-	-	-	-
23	35	Positive	L265P (8%)	-	-	-	-	-
24	40	Positive	L265P (7%)	-	-	-	-	-
25	60	Positive	L265P (27%)	-	-	-	-	-
26	30	Positive	-	-	-	-	-	-
27	10	Positive	L265P (18%)	-	-	-	-	-
28	5	Positive	L265P (17%)	-	-	-	-	-
29	5	Positive	L265P (6%)	-	-	-	-	-
30	25	Weak Positive	-	-	-	-	-	-

Supplementary Table S1 (continuation). Genomic profile of Waldenström's macroglobulinemia

Patient number	Bone marrow infiltration (%)	MYD88L265P AS-PCR status	MYD88 HTS status (VAF)	CXCR4 HTS status (VAF)	TP53 HTS status (VAF)	NOTCH1 HTS status (VAF)	SF3B1 HTS status (VAF)	ATM HTS status (VAF)
31	44	Positive	L265P (18%)	-	-	-	-	-
32	20	Positive	L265P (6%)	-	-	-	-	-
33	95	Positive	L265P (14%)	-	-	-	-	-
34	10	Positive	L265P (9%)	-	-	-	-	-
35	80	Weak Positive	-	-	-	-	-	-
36	3	Positive	-	-	-	-	-	-
37	10	Weak Positive	-	-	-	-	-	-
38	45	Weak Positive	-	-	-	-	-	-
39	<i>md</i>	Positive	L265P (22%)	-	-	-	-	-
40	<i>md</i>	Negative	-	-	-	-	-	c.998C>T;p.S333F (47%)
41	10	Negative	-	-	-	-	-	-

Abbreviations: AS-PCR, allele specific – polymerase chain reaction; *md*, missing data; HTS, high throughput sequencing; VAF, variant allelic frequency.

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TITRE DE LA THESE d'EXERCICE

Profil génomique des neuropathies anti-MAG

Rennes, le 25/08/2016

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Le Directeur de thèse

Rennes, le 29/08/16

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Rennes, le

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ALLAIN Jean-Sébastien – Profil génomique des neuropathies anti-MAG

43 feuilles, 3 figures, 5 tableaux. 30 cm.- Thèse : Médecine ; Rennes 1; 2016 ; N°

RÉSUMÉ**Introduction:**

La neuropathie anti-MAG est une polyneuropathie acquise rare associée à une IgM monoclonale produite par un syndrome lymphoprolifératif B sous-jacent. Les études génomiques ont révélé de nombreuses mutations clonales dans les hémopathies B de bas grade.

Patients et méthodes:

Les échantillons de 27 patients atteints de neuropathie anti-MAG et de 41 contrôles atteints de maladies de Waldenström (WM) ont été inclus. La mutation MYD88^{L265P} a été analysée par PCR allélique spécifique. Le statut mutationnel des gènes *MYD88*, *CXCR4*, *TP53*, *SF3B1*, *ATM* et *NOTCH1* a été étudié par un séquençage ciblé haut débit.

Résultats:

Les hémopathies associées aux neuropathies anti-MAG sont 13 gammopathies monoclonales de signification indéterminée (MGUS), 12 WM, un lymphome de la zone marginale splénique (MZL) et une lymphocytose B monoclonale. Dans ce groupe, la mutation MYD88^{L265P} est présente chez 53,8% des IgM-MGUS, 91,7% des WM et chez le MZL. Nous avons identifié respectivement 3,7%, 7,4% et 7,4% de variants dans les gènes *TP53*, *ATM* et *NOTCH1*. Aucune mutation de *CXCR4* ni *SF3B1* n'a été retrouvée. Dans le groupe contrôle de WM, MYD88^{L265P} est présente dans 95,2% des cas. Des mutations de *TP53*, *ATM* et *SF3B1* sont respectivement retrouvées chez 7,3%, 7,3% et 4,9% des contrôles. Le profil mutationnel MYD88^{L265P}CXCR4^{WHIM} (19,5%) est significativement associé au groupe WM (p<0,05). Les patients MYD88^{L265P}CXCR4^{WHIM} ont des taux d'hémoglobine et de plaquettes inférieurs ainsi qu'un envahissement médullaire plus important que les patients identifiés MYD88^{L265P}CXCR4^{wild-type} (p<0,05 pour chaque comparaison).

Discussion:

La mutation MYD88^{L265P} est fréquemment observée dans les neuropathies anti-MAG. Le profil MYD88^{L265P}CXCR4^{wild-type} est associé à cette neuropathie paranéoplasique définissant un nouveau lien entre le statut mutationnel et le phénotype clinique associée aux hémopathies B de bas-grade. Ces éléments ouvrent la porte à de nouvelles voies thérapeutiques.

Rubriques de classement :	MEDECINE INTERNE
Mots-clés :	Immunoglobuline M; Polyneuropathie paranéoplasique; Gammopathie monoclonale de signification indéterminée; Maladie de Waldenström; Facteur de différenciation myéloïde-88; CXCR4
Mots-clés anglais MeSH :	Immunoglobulin M; Paraneoplastic Polyneuropathy; Monoclonal Gammopathy of Undetermined Significance; Waldenström's macroglobulinemia; Myeloid Differentiation Factor 88; CXCR4

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