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To cite this version:

HAL Id: hal-01230365
https://hal-amu.archives-ouvertes.fr/hal-01230365
Submitted on 18 Nov 2015

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The Thymus-Specific Serine Protease TSSP/PRSS16 Is Crucial for the Antitumoral Role of CD4+ T Cells

Highlights
- TSSP is an important protease for intrathymic selection of CD4+ T cells
- Absence of TSSP favors both spontaneous and inflammation-induced tumor development
- TSSP-dependent maturation of CD4+ T cells in the thymus is required for tumor suppression
- TSSP is a thymic protease with tumor suppressor activity

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In Brief
The thymus-specific serine protease (TSSP) is involved in CD4+ T lymphocyte selection in the thymus. Brisson et al. now reveal a function of TSSP in the prevention of cancer. Both spontaneous and inflammation-associated tumor development is promoted in TSSP-deficient mice through the production of an altered CD4+ T cell compartment.
The Thymus-Specific Serine Protease TSSP/PRSS16 Is Crucial for the Antitumoral Role of CD4⁺ T Cells

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INTRODUCTION

One of the currently promising strategies in cancer treatment is immunotherapy, which consists in targeting the immune response in order to favor the elimination of cancer cells (Couzin-Frankel, 2013; Dougan and Dranoff, 2009; Mellman et al., 2011; Muranski and Restifo, 2009). During cancer development, immune cells appear to prevent the emergence of tumoral cells and inhibit cancer progression through the so-called immune surveillance (Dunn et al., 2004; Swann and Smyth, 2007). Nevertheless, immunity (both innate and adaptive) can also promote tumor development during inflammation; hence, chronic inflammatory diseases favor the growth of many human cancers (Bui and Schreiber, 2007; de Visser et al., 2006; Kundu and Surh, 2012; Trinchieri, 2012). Thus, the role of adaptive immune cells is dual, depending on the context and cell types; in particular, CD4⁺ T helper lymphocytes can either impair or favor carcinogenesis (Corthay et al., 2005; DeNardo et al., 2009; Kennedy and Celis, 2008; Ostrand-Rosenberg, 2008; Rakha et al., 2010).

Inflammatory bowel disease (IBD) patients are at increased risk of developing colorectal cancer (CRC). In CRC, on one side, T cells have been elegantly shown to be protective against cancer progression, through their antitumor effect in eliminating cancerous cells (Pages et al., 2005). On the other side, T cells participate during IBD in the protumoral inflammatory reaction stimulating uncontrolled growth of epithelial cancer cells (Monteleone et al., 2012).

We recently reported the importance of the thymus-specific serine protease (TSSP) in CD4⁺ T cell maturation in the thymus. TSSP is encoded by the PRSS16 gene, which is highly conserved between humans and mice (Bowles et al., 1999; Carrier et al., 1999, 2000). TSSP is a serine protease of yet-unknown enzymatic function, predominantly found in endosomes of thymic cortical epithelial cells. TSSP was first reported as linked to a type 1 (autoimmune) diabetes (T1D) susceptibility locus of the extended major histocompatibility complex class I region in humans (Lie et al., 1999, 2002; Viken et al., 2009). To address the function of TSSP, we generated a genetically inactivated mouse model (TSSP-deficient mice, hereafter called TSSP KO). In-depth analysis of these mice showed that they have a normal number of CD4⁺ T cells but an altered CD4⁺ T cell receptor repertoire (Gommeaux et al., 2009; Viret et al., 2011a). Moreover, the absence of TSSP on a nonobese diabetic background completely prevents the development of T1D, thus demonstrating the direct implication of TSSP in disease development (Viret et al., 2011b). Prevention of T1D development in the absence of TSSP is related to impaired thymic selection of CD4⁺ T cells specific for islet antigens (Viret et al., 2011b). Thus, our previous work evidenced a crucial role of TSSP in the shaping of a functional CD4⁺ T cell compartment. To date, the impact of TSSP in cancer has not yet been investigated. In thymic tumors,
TSSP was reported to be either over- or underexpressed, depending on the cell type affected by cancerous proliferation (cortex thymoma or thymic lymphoma, respectively) (Lin and Aplan, 2007; Strobel et al., 2014). During breeding of TSSP-deficient mice, we serendipitously observed spontaneous cancer development upon aging. In the current work, we assessed the consequence of TSSP deficiency on mouse cancer pathology and unveiled the associated mechanisms.

RESULTS AND DISCUSSION

TSSP-Deficient Mice Are Highly Susceptible to Both Spontaneous Cancer Development and Inflammation-Associated Cancer

We first addressed the question of the lifespan of TSSP-deficient mice compared to wild-type (WT) by checking mice survival during aging over a 3-year period. While the lifespan of TSSP-deficient mice did not differ from that of TSSP-proficient mice (Figure S1A), we observed spontaneous tumor development in aged TSSP-deficient mice. Indeed, 100% of TSSP KO mice died from cancer development, either hepatocarcinoma (HCC) or lymphoma (Figures S1B and S1C). C57BL/6 mice are known to rarely develop cancer, unless on an immunodeficient background (Swann and Smyth, 2007). Our observation suggests that absence of TSSP generates a permissive environment for spontaneous tumor development, potentially through a partial immunodeficiency.

In order to decipher the mechanistic basis of increased susceptibility of TSSP KO mice to cancer, we used an inflammation-induced tumorigenesis setting. The colitis-associated colorectal cancer (CAC) protocol consists of an intraperitoneal (i.p.) injection of azoxymethane (AOM), which initiates tumorigenesis, followed by three cycles of dextran sodium sulfate (DSS) in the drinking water to induce a moderate, long-lasting colitis. (A) Scheme of the CAC (AOM DSS) protocol. Colons are collected 10 weeks after injection of AOM and analyzed for the presence of tumors. (B) Mean number ± SD of tumors and their size distribution per colon from WT and TSSP-deficient (KO) animals (**p < 0.01). (C) Mean ± SD of the weight-to-length ratios of the colons (mg/mm). The dashed line corresponds to the mean weight-to-length ratio of control animals (**p < 0.001). The total number of animals is combined from three independent experiments, and data are representative of two different experiments. (D and E) Overview of hematoxylin and eosin-stained sections of representative colons from WT (D) and TSSP-deficient mice (E). Representative tumors are shown for each genotype. Scale bar corresponds to 100 μm.

More Severe Inflammation in TSSP-Deficient Mice Than in WT Likely Accounts for Increased Colitis-Associated Tumorigenesis

As inflammation is a major factor of CAC development, we induced acute colitis by adding 3.5% DSS in the drinking water of TSSP KO and WT mice for 7 days. We observed higher body weight loss, mortality, disease activity index (which combines stool consistency, rectal bleeding, and weight loss), and mucosal ulcerations in TSSP-deficient as compared to TSSP-proficient mice (Figure 2). Thus, DSS-induced acute colitis is more severe in TSSP-deficient as compared to TSSP-proficient mice. This result suggests that regulation of acute inflammation is altered in the absence of TSSP, proposing a role for TSSP in inflammation control. Exacerbated DSS-induced chronic inflammation during the CAC protocol could explain increased colorectal tumors development in the absence of TSSP.

T Cells Play a Key Role in Increased CAC in the Absence of TSSP

The increased CAC development in TSSP-deficient mice could result from a defect in either epithelial cells (which give rise to
colorectal tumors) or from immune system function (which is involved in epithelial barrier homeostasis). We previously reported that the Prss16 gene encoding TSSP in mouse is highly expressed in cortical thymic epithelial cells, a feature also reported in humans (Bowlus et al., 1999; Carrier et al., 1999). We investigated Prss16 expression in other tissues, including colon, by quantitative RT-PCR experiments. Contrary to thymic stroma, where TSSP expression was very high, very little mRNA was detected in liver (both hepatocytes and nonparenchymatous immune cells), spleen, macrophages, and colon intraepithelial cells of unchallenged or CAC (AOM DSS) mice (Figure S2A). Thus, we propose that TSSP expression is specific to the thymic stromal microenvironment and postulate that higher CAC susceptibility of TSSP-deficient mice stems from immune cell defects acquired during thymic development rather than from colon epithelial cell deregulation.

To investigate the specific role of immune cells in increased CAC susceptibility of TSSP-deficient mice, we used immunodeficient mice. We crossed TSSP KO with CD3 epsilon delta 5 (named here CD3 KO)-deficient mice, which are specifically devoid of T lymphocytes (Malissen et al., 1995). Development of CAC was similar in CD3 KO mice as compared to WT mice (Figure 3A). In addition, contrary to what was observed in TSSP KO mice as compared to WT, the number of CAC tumors was not increased in double-KO mice as compared to CD3 KO (Figure 3A). These data suggest that the role of TSSP on CAC is mediated by T lymphocytes as initially postulated. To formally demonstrate this hypothesis, we performed T cell adoptive transfer experiments in CD3 KO recipient mice. The presence of T cells in CD3 KO recipients was checked during the course of, and at the end of, the experiment (Figure S2B). Data reported in Figure 3B show that (1) the presence of T cells in CD3 KO recipient mice increases the number and size of CAC tumors and (2) development of CAC tumors is higher when the injected T cells were purified from TSSP KO mice than from WT.

As cytokines are primary molecular mediators of immune cells that can be either protective or permissive in CAC (Waldner and Neurath, 2009), we monitored the expression of Th cytokines (interleukin-10 [IL-10], IL-4, interferon [IFN]-γ, IL-17A) in the colon of mice by quantitative RT-PCR. Interestingly, the levels of IFN-γ (the signature of antitumoral Th1 cells) and anti-inflammatory IL-10 are lower in TSSP-deficient mice than in WT mice (Figure 3C, left). In contrast, level of IL-4 (the signature of protumoral Th2 cells) is high in CD3 KO recipient mice injected with TSSP KO T cells (Figure 3C, right). Strikingly, the expression level of protumoral IL-17A is high when T cells are deficient for TSSP both in a CD3 WT background (Figure 3C, left) and in a CD3 KO background upon adoptive transfer (Figure 3C, right). Taken together, these data show that absence of TSSP is associated with a protumoral Th cytokine profile.

**CD4+ T Cells Are Pivotal in Increased CAC in the Absence of TSSP**

We previously demonstrated that TSSP is involved in the maturation of a subset of CD4+ T cells (Gommeaux et al., 2009; Viret et al., 2011a, 2011b). We therefore sought to gain further insight into the role of CD4+/CD8+ T cells in CAC development by performing adoptive transfer experiments using a mixture of CD4+ and CD8+ T cells (purified from either TSSP KO or WT mice) injected in CD3 KO recipient mice. As observed in the preceding experiment, the presence of T cells in CD3 KO mice gave rise to increased numbers of CAC tumors (Figure 3D). Strikingly, when CD4+ T cells were purified from TSSP KO mice, the number and size of CAC tumors were much higher than with CD4+ cells.
Inhibition of IL-17A Prevents the Increase in CAC in the Absence of TSSP

In order to assess the increased protumoral action of IL-17 in TSSP-deficient colons, we treated mice with anti-IL-17A antibodies weekly during the CAC protocol. As a control, mice injected with control isotype antibodies showed higher colon tumor numbers and size when deficient for TSSP as compared to WT, as is the case in all CAC experiments (Figure 4A). Remarkably, inhibition of IL-17A not only reduced tumor burden in the two genotypes but also almost completely erased the difference between both genotypes. This result strongly supports the key role of IL-17A overexpression in exacerbated CAC in the absence of TSSP. We monitored the expression of cytokines in CAC colons in isolated tumoral and nontumoral (healthy) areas (Figure 4B). The main information provided by these experiments is that (1) in control KO colons (control-isotype treated), anti-inflammatory cytokines (IL-10 and IFN-γ) are reduced whereas proinflammatory cytokines (IL-4 and IL-17A) are increased compared to WT, which is consistent with data shown in Figure 3; (2) in control KO colons, the level of IL-4 and IL-17A is higher in tumoral areas than in nontumoral areas; and (3) most importantly, differences between KO and WT colons are reduced or completely abolished when mice are treated with anti-IL-17A antibody. In parallel, we analyzed immune cells in secondary lymphoid organs by flow cytometry (Figure 4C). No differences were observed between the two genotypes, except for a significant decrease in the number of T regulatory (Treg) cells in the spleen of control-isotype-treated CAC KO mice, which is abolished upon anti-IL-17A treatment. This observation is interesting, since Treg cells are known to play an antitumoral role in CAC by...
dampening inflammation, in contrast with their more famous protumoral role by attenuating tumor immunosurveillance (Waldner and Neurath, 2009). We also analyzed the same immune cells in lymphoid organs from nonchallenged mice at different ages and observed no differences, as previously reported (Gommeaux et al., 2007; Viret et al., 2011b). Taken together, those data show that neutralization of IL-17A alleviates the CAC susceptibility of TSSP-deficient mice by dampening cytokinic immune disequilibrium in the colon.

To better characterize the colon immune disequilibrium associated with TSSP deficiency, we analyzed immune cells in the colon. We first analyzed lamina propria lymphocytes (LPLs) in the colons of nonchallenged mice and observed no differences between the two genotypes (data not shown). In contrast, the immune cell content (LPLs + tumor-infiltrating lymphocytes [TILs]) of CAC-induced colons differed between genotypes (Figure S4).

![Figure S4A](image)

**Figure S4.** Inhibition of IL-17A Cytokine Rescues the Phenotype of TSSP-Deficient Mice

Impact of IL-17A has been evaluated by the injection of either control isotype or blocking anti-IL-17A antibody during the course of CAC development.

(A) CAC tumor mean number ± SD and size distribution per colon depending on mice genotype and antibody injection (n = 5–9). Differences between TSSP-deficient and WT mice are almost completely abolished when mice are injected with anti-IL-17A antibody. *, $, and £, compared to control WT, control TSSP KO, and anti-IL-17A-treated WT mice, respectively.

(B) Expression of cytokines (mean ± SD) in sane and tumoral regions of colons was evaluated by quantitative RT-PCR and compared between TSSP-deficient and WT mice injected with either control or anti-IL-17A antibody. From one to three, marks indicate p values less than 0.05, 0.01, and 0.001, respectively.

(C) Immune cell percentages (mean ± SD) in secondary lymphoid organs evaluated by flow cytometry. Except for splenic Tregs (*p < 0.05), no differences were observed between WT and TSSP-deficient mice.

Data are representative of two independent experiments.
of CAC colons (Figure S4B). In WT colons, immune cells are spread throughout the whole colon (except granulocytes, which are enriched in areas containing tumors, as is the case in KO mice). In contrast, in KO colons, tumoral regions contain less CD8+ T cells and more Tregs than healthy areas. Most importantly, cancer colons contained numerous IL-17-secreting immune cells (not only CD4+ Th17 cells), with an increase of those cells in the healthy (peritumoral) areas of KO colons. These data are consistent with immunohistofluorescence data (Figure S3B), which nicely show a decrease in CD8- T cell numbers in KO colon tumors and prominent accumulation of IL-17+ cells in peritumoral areas of KO colons. IL-17A is known to be secreted by several cell types (Th17, γδ T, innate lymphoid cells, natural killer T cells, and neutrophils), which all play a role in carcinogenesis (De Simone et al., 2013). Altogether, these data show that both the percentage of different immune cell types and their tissue localization are affected in the colon of TSSP-deficient mice in the CAC context, showing that TSSP deficiency is associated with immune response disequilibrium during CAC, thus generating an unfavorable “antitumor immune contexture” (Fridman et al., 2012). This defect is rescued by inhibition of IL-17A, thus demonstrating the prominent role of IL-17 in high CAC susceptibility in a TSSP-deficient immune environment. This study therefore further stresses the crucial role of IL-17-producing cells in colorectal cancer development and contributes to the elucidation of immune mechanisms.

In conclusion, this work demonstrates that the cancer susceptibility of TSSP KO mice results from an altered CD4+ T cell compartment and loss of cytokine balance. In CAC, the absence of TSSP is associated with increased protumoral IL-17 responses. Interestingly, the Th17 response has been associated with promotion of colorectal development in mice and a poor prognosis in colorectal cancer patients (Tosolini et al., 2011; Wu et al., 2009). The colonic commensal microflora has been shown to play a major role in promoting expression of IL-17A, which triggers colorectal tumorigenesis (Grivennikov et al., 2012). It has been proposed that development of tumor-infiltrating CD4+ Th17 cells may be a general feature in cancer patients (Su et al., 2010). Our data point to the contribution of other IL-17 producing cells. Therefore, this work furthers our general understanding of the role of immune cells in the control of colorectal cancer development, which needs to be taken into consideration in clinical oncology. In addition, this work provides a valuable preclinical mouse model for the development of new immunotherapy strategies.

**EXPERIMENTAL PROCEDURES**

**Mice**

Generation of TSSP-deficient (Prss16<−/−>) mice backcrossed on the C57BL/6 parental genetic background and their genotyping by PCR were described previously (Gommeaux et al., 2009). Mice deficient for the TCR subunit CD3δ (CD3δ δelta5 mice devoid of T lymphocytes, referred as CD3 KO in the text) were a kind gift of Marie Malissen (Malissen et al., 1995). Male mice entered protocols at 8 weeks of age except for adoptive transfer (4 weeks old). All mice were kept within the animal facilities and according to the policies of the Laboratoire d’Exploration Fonctionnelle de Luminy (Marseille, France).

**Tumor Induction**

CAC tumors were induced as previously reported (Gommeaux et al., 2007). Briefly, TSSP-deficient male mice and WT littermates (8–10 weeks old) were injected i.p. with 12.5 mg/kg AOM (Sigma). After 5 days, 2.5% DSS (MP Biomedicals; molecular weight = 36,000–50,000 Da) was given in the drinking water over 5 days, followed by 16 days of tap water. This cycle was repeated twice (5 days of 2.5% DSS and 4 days of 2% DSS), and mice were sacrificed 10 days after the last cycle. When specified, mice were intravenously (i.v.) injected with 2 μg of either anti-IL-17A antibody (LEAF purified anti-mouse IL-17A from BioLegend) or control isotype (LEAF purified rat IgG1, κ isotype control from BioLegend) weekly during CAC protocol.

**Induction and Analysis of Acute Colitis**

Treatment with DSS leads to acute colonic inflammation with superficial ulceration, mucosal damage, and leukocyte infiltration. DSS is toxic to mucosal epithelial cells, the eventual dysfunction of the mucosal barrier leading to mucosal inflammation. Male mice (7–10 weeks old) were given 3.5% DSS in their drinking water for 7 days, followed by water, only until sacrifice. Every day during and after DSS administration, mice were monitored for body weight loss, pathological features (rectal bleeding and diarrhea), and survival. Body weight was expressed as a percentage of the initial weight on day 0 of the protocol. Presence of diarrhea, rectal bleeding, and weight loss were separately graded on a 0–3 scale (Gommeaux et al., 2007), and the sum of the three values constitutes the disease activity index.

**Morphological and Histological Analyses**

Colons were removed, rinsed free of feces with PBS, cut open longitudinally, carefully dried on blotting paper, weighed, measured, and photographed at high resolution. For each colon individually, weight-to-length ratio was determined, in milligrams per millimeters of colon. For histochemical analysis, colon samples were fixed flat in 4% formaldehyde at 4°C overnight and paraffin embedded as "Swiss rolls" containing the full-length organ. Five-micrometer-thick sections were stained with hematoxylin and eosin for histopathological assessment of colitis and tumors. Integrity of mounted stained sections surfaces were viewed on a Nikon microscope, and representative pictures are shown. Proliferation (Ki67 immunostaining) and immunohistofluorescence analyses were performed as described in Supplemental Experimental Procedures.

**Adaptive Transfer and Flow Cytometry**

For adoptive transfer, total T (CD3+) cells, CD4+ cells, or CD8+ cells were purified from spleens by negative selection using EasySep negative selection kits (STEMCELL Technologies). Four-week-old CD3 KO recipient male mice were i.v. injected with 10–15 × 10⁶ T cells (2:1 ratio when mixture of CD4+ and CD8+ cells) isolated from 8- to 10-week-old male donors. Blood cells were analyzed 3 days after adoptive transfer by staining with CD4, CD8α, and CD3ε specific antibodies in fluorescence-activated cell sorting (FACS) buffer (PBS/3%/fetal calf serum [FCS]), and the CAC protocol was started the week after. At the end of the CAC protocol, lymphocyte suspensions were prepared from spleens and lymph nodes and stained with the specified antibodies (BD Biosciences or BioLegend) after blocking of FcR by incubation for 15 min at 4°C and then analysis using a FACS caliber flow cytometer (BD Biosciences), and data analysis was performed using FlowJo software (Tree Star).

**Purification and Analysis of Lamina Proprria Lymphocytes (LPLs)**

Purification of LPLs was performed as described previously (Weigmann et al., 2007). Briefly, colons were cut into 0.5 cm pieces, washed in Hank’s balanced salt solution (HBSS)/2% FCS, then incubated in HBSS/2 mM EDTA at 37°C under rotation twice (15 min then 30 min). Pieces were filtered on 70 μm cell strainer then incubated in RPMI/10% FCS/1% HEPES containing 1 mg/ml collagenase 8 (Sigma) at 37°C under rotation for 45 min or 1 hr when colons contained tumors to favor the liberation of TILs. Cells were passed through a 70 μm cell strainer then isolated by centrifugation with 40/90 Percoll (Sigma) gradient for 20 min at 1,000 × g at 20°C without braking. Cells were then resuspended into FACS buffer and stained with fluorochrome-labeled antibodies (BioLegend) specific for immune cells markers (CD3+CD8+CD4+ for T cells, CD4+FoxP3+IL-17A for Tregs and Th17, Gr1+CD11b for granulocytes) after blocking of FcR/III/II. For intracellular staining (nuclear FoxP3 or cytoplasmic IL-17A), cells were fixed and permeabilized using Fix/Perm and Perm/Wash (Life Technologies). Stained cells...
were analyzed on a MACSQuant VYB flow cytometer (Miltenyi Biotec), and data analysis was performed using FlowJo software (Tree Star). Lymphoid cells were gated on forward scatter/side scatter dot plots for CD4, CD8, and Treg cell analysis; total viable cells were gated for granulocytes and IL-17 cell analysis.

**Expression Analysis**

Total RNA was isolated from frozen tissue samples with TRizol reagent (Life Technologies) according to the manufacturer’s instructions. Reverse transcription of total RNA (2–5 μg) was realized using GoScript Reverse transcription system with provided oligodeoxynucleotide primers (Promega). cDNA amplicons were amplified with specific primers (Table S1) and GoTaq qPCR Master Mix kit (Promega) using a Mx3000P Stratagene device. For cytokine expression analysis, each data point represents results obtained from at least five entire colons made in triplicate.

**Statistical Analyses**

Results are expressed as the mean ± SD of results from at least two independent experiments. Statistical analyses were performed via Student’s t test.

**Study Approval**

Care and manipulation of mice were performed in accordance with national and European legislation on animal experimentation and were approved by the Aix-Marseille University Institutional Animal Care and Use Committee.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.12.009.

**AUTHOR CONTRIBUTIONS**

L.B. and A.C. designed and carried out the experiments and wrote the manuscript. L.P. designed and carried out colitis and CAC experiments. N.L. carried out immunological experiments. G.W. performed the experiments on aged mice. S.G. performed tumors histological analyses. N.L. carried out immunological experiments. G.W. performed the adoptive transfer experiments. All authors discussed the results and commented on the manuscript.

**ACKNOWLEDGMENTS**

We are grateful to Fabrice Gianardi for assistance with the use of the Laboratoire d’Exploration Fonctionnelle de Luminy animal housing facility; Marie Malissen (CIML) for CD3 KO mice; Marie-Noëlle Lavaut, Marion Rubis, and Lionel Chasson (Histology Platform of CIML) for support in histological analysis; Laurence Borge for help in flow cytometry; Noushine Mosaadegh-Keller (CIML) for support in intravenous injections; and Sylvie Guerder, Christophe Viret, Mathias Chamaliard, and Jean-Pierre Couty for helpful discussions. We also thank Valérie Depraetere-Ferrier for editing the manuscript. The authors are supported by Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Institut National du Cancer (INCa), Cancéropole PACA, Association pour la Recherche sur le Cancer (Fondation ARC), and Agence Nationale pour la Recherche (ANR). L.B. was supported by ANR, L.P. by Fondation pour la Recherche Médicale, and P.N’g. by INCa and La Ligue Nationale contre le Cancer.

Received: February 27, 2014
Revised: October 30, 2014
Accepted: December 4, 2014
Published: December 24, 2014

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