Analysis of $p27^{Kip1}$ expression in insulinomas developed in pancreatic β-cell specific Men1 mutant mice

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Abstract

Multiple Endocrine Neoplasia type 1 (MEN1) is a hereditary disease characterised by the occurrence of multiple endocrine tumours. The biological functions of the responsible gene, MEN1, and its encoded protein, menin, remain so far largely elusive. The recent generation of Men1 mutant mice by our group and other laboratories provides powerful tools allowing for the identification of cellular and molecular events that occur after gene disruption. Interestingly, it has been recently reported that $p27^{Kip1}$ expression is regulated by menin and that decreased $p27^{Kip1}$ expression can be found in MEN1 insulinomas and parathyroid adenomas. In order to address whether and when $p27^{Kip1}$ expression alters during insulinoma development in pancreatic β-cell-specific Men1 mutant mice, we analysed $p27^{Kip1}$ expression in islet lesions from mutant mice at different ages. Our data revealed that $p27^{Kip1}$ protein expression was reduced in 40 out of 52 (77%) insulinomas analysed, whereas the remaining 12 insulinomas (23%) did not show altered $p27^{Kip1}$ expression. No difference between the insulinomas with and without decreased $p27^{Kip1}$ expression could be observed in terms of histological features or menin inactivation. Furthermore, our analysis on hyperplastic and dysplastic islets developed in young mutant mice showed the lack of detectable alteration in $p27^{Kip1}$ expression, despite evident loss of menin expression in a substantial proportion of islet cells. Our work confirms the altered $p27^{Kip1}$ expression reported in tumours from MEN1 patients, whereas it suggests that other molecular events may also participate in the tumorigenesis process initiated by the Men1 gene inactivation.

Abbreviations: Cre: causes recombination; MEN1: multiple endocrine neoplasia type 1; Rip: rat insulin promoter

Introduction

Multiple Endocrine Neoplasia type 1 (MEN1) is a hereditary syndrome transmitted with an autosomal dominant trait, characterised by the occurrence of multiple endocrine tumours of the parathyroids, pancreas, and anterior pituitary [1, Online Mendelian Inheritance in Man No. 131100]. Analyses carried out by different laboratories detected germline mutation of the MEN1 gene in about 70–90% of familial MEN1 patients [2–4]. Somatic mutations were also found in a substantial proportion of sporadic endocrine tumours, especially in insulinoma, gastrinoma and parathyroid adenoma [5, 6], whereas the frequency of mutations found in sporadic pituitary and adrenal adenomas was extremely limited [7, 8]. It is noticed that the mutations revealed in the above analyses showed a typical ‘loss of function’ profile, establishing no genotype–phenotype correlation. The loss of heterozygosity frequently observed in MEN1 tumours supports the hypothesis that the MEN1 gene acts as a tumour suppressor.
Biochemical studies have identified several binding partners for menin, encoded by the MEN1 gene, including JunD [9], Smad1, 3 and 5 [10, 11], nm23 [12], Pem [13], and NF-κB family components p50, p52 and p65 [14]. The fact that menin interacts physically and functionally with several transcriptional factors suggests that the protein may be involved in the regulation of transcription. Indeed, several genes have recently been identified as gene targets whose expression is regulated by menin, including telomerase [15], insulin [16], prolactin [17] and the MEN1 gene itself [18]. The identification of the new partners of menin, particularly RPA2 [19], mSinA3 [20], and MLL1 and MLL2, the members of the Set1 protein complex [21, 22] capable of methylating lysine 4 of histone H3, reinforces this hypothesis. However, the current knowledge on these menin partners does not allow us to explain pertinently either the occurrence of MEN1 disease, or the biological function of menin.

To study the role of the MEN1 gene in endocrine malignancy, we and others have generated Men1 mutant mice using either the conventional or conditional gene targeting strategy [23–25]. The heterozygous Men1 mutant mice start, at around 12 months of age, to develop major endocrine tumours seen in MEN1 patients, whereas the homozygous Men1 mutant embryos die at E11.5–E13.5 with multiple developmental defects. In parallel, pancreatic β-cell-specific and parathyroid-specific MEN1 gene disruption result in the development of insulinoma and parathyroid adenoma, respectively [26–28]. We believe that these Men1 mutant mouse models constitute powerful tools for the study of the mechanisms involved in tumour development. In particular, we have noticed that the insulinoma developed in β-cell-specific Men1 mutant mice, but also relatively synchronised [26], making such mice a relevant model for dissecting the disturbed biological functions and deregulated molecular events that occur during the tumour initiation and progression.

Recently, it was reported that menin directly regulates expression of the cyclin-dependent kinase inhibitors p27<sup>kip1</sup> and p16<sup>ink4a</sup>, known to play a central role in controlling cycle regulation, by recruiting MLL to their promoter [29]. The authors also demonstrated that islet tumours from MEN1 patients showed decreased p27<sup>kip1</sup> expression compared with normal endocrine tissues. To know whether the same molecular event is conserved in mice, and in particular, to find out when p27<sup>kip1</sup> expression alters during the tumour development, we have analysed p27<sup>kip1</sup> protein expression in above-mentioned pancreatic β-cell-specific Men1 mutant mice at different ages. Our data demonstrate reduced p27<sup>kip1</sup> expression in a substantial proportion of analysed insulinomas. Furthermore, p27<sup>kip1</sup> expression does not seem to be changed in early islet lesions where menin inactivation can be readily found.

Materials and methods

Men1<sup>F/F–RipCre<sup>+</sup></sup> pancreatic β-cell-specific Men1 mutant mice

Pancreatic β-cell-specific Men1 mutant mice were generated by crossing homozygous mice carrying the floxed Men1 allele (Men1<sup>F/F</sup>) with RipCre transgenic mice expressing Cre under the control of the rat insulin promoter, termed Men1<sup>F/F–RipCre<sup>+</sup></sup> mice [26].

Histological and immunohistochemical analysis of pancreatic tissues

Pancreases were collected, fixed and analysed as described [26]. Histopathological analysis was carried out on 3 μm sections stained with hematoxylin–eosin (H&E). Immunohistochemical staining was performed as described previously [26] using antibodies against menin (C19, polyclonal, 1:500, Santa-Cruz Biotechnology, California, USA), and p27<sup>kip1</sup> (C19, polyclonal, 1:500, Santa-Cruz Biotechnology).

Isolation of mouse pancreatic islet

Pancreatic islets were isolated from mice according to the protocol previously described [26]. Briefly, 3 ml of 1 mg/ml collagenase (SERVA-17451) in Hank’s Buffered Saline Solution (HBSS) was injected into pancreas through the bile duct. Pancreases were then removed and incubated at 37 °C for 20 min, and dissociated by mechanical pipetting. Islets were ‘hand-picked’ from dark field dishes under a dissecting microscope and pooled for further analysis.

Protein extract preparation and Western blotting

Enriched nuclear protein fractions from islets were prepared and analysed by Western blotting as described previously [30]. Normal islets from several wild type mice were further pooled for protein extraction because of limited protein content. The primary antibodies used were raised against menin (C19, 1:7500), p27<sup>kip1</sup> (C19, 1:1000) and actin (monoclonal, 1:20,000, ICN, Aurora, USA).

Results

Our previous data revealed that the disruption of the Men1 gene could be evidenced in pancreatic islets in Men1<sup>F/F–RipCre<sup>+</sup></sup> mice as early as 1 month of age, whereas the development of insulinoma in these mice could only be observed starting from around 6 months of age [26, also see Figure 4]. The existence of such a latent period may indicate that the disruption of the Men1 gene is essential but insufficient for the development of insulinoma. Other genetic and epigenetic factors may be needed for the process of tumour development.