European Journal of Cancer 91 (2018) 125–135

Available online at www.sciencedirect.com

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journal homepage: www.ejcancer.com

Original Research

Histamine H4 receptor as a novel therapeutic target for the treatment of Leydig-cell tumours in prepubertal boys

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Received 20 July 2017; received in revised form 18 October 2017; accepted 7 December 2017

KEYWORDS
Histamine;
Leydig-cell tumour;
Histamine receptor H4;
CYP19 aromatase;
Oestradiol;
Angiogenesis;
Steroidogenesis

Abstract Leydig-cell tumours (LCTs) are rare endocrine tumours of the testicular interstitium, with recent increased incidence. Symptoms include precocious puberty in children; and erectile dysfunction, infertility and/or gynaecomastia, in adults. So far, scientific evidence points to aromatase (CYP19) overexpression and excessive oestrogen and insulin-like growth factor (IGF) −1 production as responsible for Leydig-cell tumourigenesis. LCTs are usually benign; however, malignant LCTs respond poorly to chemo/radiotherapy, highlighting the need to identify novel targets for treatment. Herein, we investigated the potential role of the histamine receptor H4 (HRH4) as a therapeutic target for LCTs using R2C rat Leydig tumour cells, a well-documented *in vitro* model for Leydigioma. Also, we studied for the first time the expression of CYP19, IGF-1R, oestrogen receptor (ER) α, ERβ, androgen receptor (AR) and HRH4 in human prepubertal LCTs *versus* normal prepubertal testes (NPTs). HRH4 agonist treatment inhibited steroidogenesis and proliferation in R2C cells and also negatively affected their pro-angiogenic capacity *in vitro* and *in vivo*, as assessed by evaluating the proliferative activity of human umbilical vein endothelial cells and by means of the quail chorioallantoic membrane assay, respectively. Moreover, E2 and IGF-1 inhibited HRH4 mRNA and protein levels. In human prepubertal LCTs, CYP19, IGF-1R, ERα and ERβ were

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https://doi.org/10.1016/j.ejca.2017.12.003

overexpressed compared with NPTs. In contrast, HRH4 staining was weak in LCTs, but moderate/strong and confined to the interstitium in NPTs. Importantly, HRH4 was absent or barely detectable in seminiferous tubules or germ cells. Overall, our results point to HRH4 as a novel therapeutic target in LCTs.

1. Introduction

Leydig-cell tumours (LCTs) are steroid-secreting tumours of the testicular interstitium, which affect males at any age with two peaks of incidence: during prepuberty, between 5 and 10 years and in adulthood between 25 and 35 years [1]. Clinical manifestations most commonly include virilising syndromes in prepubertal boys and loss of libido, erectile dysfunction, infertility, gynaecomastia, feminine hair distribution and/or gonadogenital atrophy in adults [2,3]. LCTs are not exclusive of the male gonad because they can also occur in the adrenal gland, ovary and spermatic cord [4,5]. Although LCTs are usually benign, about 10% become malignant and do not respond to radiotherapy or chemotherapy [3]. Current literature on appropriate clinical management is limited, given the rarity of these tumours, and their treatment is mainly based on clinical reports, highlighting the need to identify new therapeutic targets for treatment [3]. To complicate the picture, the testis has been shown to be highly susceptible to the toxic effects of radiation and chemotherapy at all stages of life, being the prepubertal testis most vulnerable because of its constant turnover of early germ cells and the maturation of the Leydig-cell pool and other somatic compartments [1,6,7].

Although the precise aetiology of LCTs is still unknown, experimental findings in animal and cell-line models, as well as clinical observations in adult patients, indicate that overexpression of aromatase (CYP19) and excessive oestrogen production play a significant role in sustaining Leydig-cell tumourigenesis [2]. Also, enhanced expression of several components of the insulin-like growth factor-1 (IGF-1) signalling pathway and a cooperation between E2 and IGF-1 have been reported in R2C Leydig tumour cells as well as in an *in vivo* LCT model [8]. So far, no reports are available on human prepubertal LCTs.

The histamine (HA) H4 receptor (HRH4) is currently considered a promising drug target for allergy, inflammation, autoimmune disorders and cancer, as reflected by a steadily growing number of scientific publications and patent applications [9]. Previously, we reported that HRH4 agonist treatment inhibits both Leydig-cell proliferation and Luteinizing hormone / human chorionic gonadotropin (LH/hCG)-induced steroidogenesis in MA-10 Leydig tumour cells [10]. Herein, we investigated the potential role of HRH4 as a therapeutic target for LCTs using the R2C Leydig tumour cell line, the best-known *in vitro* model for Leydigioma, in which CYP19 has been found to be overexpressed [8]. Also, in view of the very limited knowledge regarding LCTs in boys, we assessed for the first time the expression of CYP19, IGF-1R, oestrogen receptor (ER) α, ERβ and androgen receptor (AR), as well as HRH4, in human prepubertal LCTs *versus* normal prepubertal testes.

2. Materials, patients and methods

2.1. Materials

Materials and their respective suppliers are listed in Supplementary Data.

2.2. Patients

Clinical information of the patients is summarised in Supplementary Table 3.

2.3. Culture of R2C Leydig tumour cells and HUVEC cells

The R2C rat Leydig tumour cell line was purchased from American type culture collection (ATCC) (# 58649146), whereas human umbilical vein endothelial cells (HUVECs) were a generous gift from Dr. Alberto Baldi (Laboratorio de Patología y Farmacología Molecular, IBYME-CONICET, Buenos Aires, Argentina). Experiments with both cells lines were performed in triplicate, with at least three different cell-line batches or passage numbers (<12). See Supplementary Data for details on cell culture.

2.4. Western blot analysis and immunodetection of proteins

Cells were treated as indicated in each figure for 8 h. Protein extraction, Western blotting and quantitation of immunospecific bands were performed as described [10].