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Localization of insulin-like growth factor-II mRNA in human pituitary adenomas

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Abstract Insulin-like growth factors (IGFs) have been reported to promote cell proliferation in many tumours, but their contribution to pituitary adenoma development and growth has not been characterized. We report the presence of insulin-like growth factor II (IGF-II) mRNA in pituitary adenomas using in situ hybridization (ISH). The intensity of IGF-II hybridization signal was correlated with adenoma type, and the presence of Ki-67. Among the 109 adenomas examined, 55 (50.4%) were positive for IGF-II mRNA. All acidophil stem cell, functioning corticotrophic and plurihormonal adenomas contained the message; a high incidence of signal was found among sparsely (7/8) and densely (4/6) granulated growth hormone (GH) cell adenomas, mixed GH cell–prolactin (PRL) cell adenomas (6/7), thyrotrophic (4/6) and null-cell (6/7) adenomas. Less frequently, IGF-II mRNA was localized in mammosomatotrophic, silent subtype 3, gonadotrophic, and oncocytic adenomas, whereas all sparsely granulated PRL cell adenomas and silent corticotrophic adenomas of subtypes 1 and 2 were negative. The MIB-1 labelling index was significantly higher in adenomas with a moderate to intense IGF-II signal than in adenomas with weak or no signal. The results suggest that IGF-II, when highly expressed, may have a role in pituitary adenoma proliferation.

Key words Insulin-like growth factor-II · Human pituitary · Neoplasm · Cell proliferation · Pathology

Introduction

Insulin-like growth factors (IGF-I and IGF-II) act as regulating hormones and locally produced growth factors via specific receptors, eliciting responses in an endocrine, autocrine or paracrine manner. Extensive in vivo and in vitro studies have documented that circulating IGF-I originating in liver is a negative regulator of pituitary growth hormone (GH) production. The action of IGF-II is less well defined. IGFs are produced in most tissues, including pituitary, with variations depending on age and cell type. In general, IGF-I has been considered to have a primary role in the postnatal period and whereas IGF-II, during embryonic and fetal life [11]. However, studies in mice with inactive IGFs have demonstrated that both IGF-I and IGF-II have important roles in fetal development [2, 8, 9, 24, 27]. Type-1 IGF receptor mediates the signalling of both growth factors, while type-II IGF receptor and XR (still unidentified receptor) serve for IGF-II [2, 22, 30]. It seems that pituitary IGF-II receptor does not play any part in GH gene regulation, but may be involved in the sequestration of IGF-II and other uncharacterized IGF-II mediated functions [39].

IGF-II peptide has been detected in the endocrine cells of the pars distalis and pars intermedia of the ovine pituitary [25]. In rat, IGF-II mRNA is abundant in neural and intermediate lobe capillaries, and low levels are present in endocrine cells of the anterior and intermediate lobes [1]. In human anterior pituitary, large amounts of IGF-II have been demonstrated by radioimmunoassay [16]; however, the localization of IGF-II and its mRNA has not been demonstrated by immunohistochemistry and in situ hybridization (ISH).

IGF-II is constitutively expressed in many tumour types and has been shown to be mitogenic for breast cancer cell lines, mouse pancreas tumours and human colon tumours [7, 40, 41]. The stimulation of tumour growth is based on the activation of IGF-I R, since activation of IGF-II R is not mitogenic [3]. Transgenic mice with elevated IGF-II blood levels develop a diverse spectrum of tumours at advanced ages at a higher rate than control mice [28]. These findings suggest that IGF-II may function as a tumour progression factor.

Significantly increased levels of IGF-I and IGF-II measured by radioimmunoassay have been found in the cerebrospinal fluid (CSF) of patients with brain tumours and pituitary adenomas, suggesting that IGFs are re-

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leased by the tumour cells [12]. However, the localization and role of IGF-II in human pituitary adenomas have not been documented. We have studied the presence of IGF-I mRNA in pituitary adenomas [35]. IGF-I A and B mRNAs, the two alternatively spliced variants, have been detected in almost all cases, including all types of adenomas. The significance of widespread distribution and overexpression of IGF-I, especially of variant B transcripts in pituitary adenomas, is unknown.

We document the presence of IGF-II mRNA in non-neoplastic human pituitary and different types of adenohypophysial adenomas by an ISH method. The distribution of IGF-II transcripts in different adenomas is correlated with the presence of Ki-67, a tumour proliferation marker.

Materials and methods

Pathological Material

Ten non neoplastic pituitaries, 7 from autopsy cases and 3 obtained at surgery, were investigated. The presence of IGF-II mRNA was assessed in 109 pituitary adenomas obtained at surgery: 6 densely granulated growth hormone (GH) cell adenomas, 8 sparsely granulated GH cell adenomas, 6 sparsely granulated prolactin (PRL) cell adenomas, 7 mixed GH cell–PRL cell adenomas, 7 mammosomatotrophic adenomas, 5 acidophil stem cell adenomas, 6 functioning corticotrophic adenomas, 7 silent corticotrophic adenomas (3 subtype 1, 4 subtype 2), 8 silent subtype 3 adenomas, 22 gonadotroph adenomas (female and male types), 6 thyrotrophic adenomas, 7 null-cell adenomas, 8 oncocytomas, and 6 plurihormonal adenomas. Tumours were characterized histologically and immunohistochemically, and classified ultrastructurally.

ISH was carried out on 5-μm deparaffinized sections using a radiolabelled oligodeoxynucleotide probe for IGF-II. The probe complementary to bases 607–645 of human IGF-II mRNA was purchased from DuPont Canada, (Lachine, Quebec, Canada). It was labelled by a 3'-end method with [^{35}S]dATPαS and terminal deoxynucleotidyl transferase using a kit (NEP-100, Dupont Canada), and purified with NENSORB 20 cartridge.

The details of prehybridization and hybridization treatments of the sections have already been described in detail [34]. The concentration of IGF-II oligoprobe was 1.0–2.0 × 10^6 cpm/slide. Controls for ISH included predigestion of sections with 100 μg/ml RNase (Sigma) at 37°C for 30 min, and competition studies with 100-fold excess of unlabelled probe.

Semiquantitative evaluation of the IGF-II mRNA signal intensity was performed by counting the number of silver grains with 100× objective over an area covering a minimum of 30 nuclei in each case; a mean signal was determined for each case. Silver grains were also counted in RNase-pretreated slides to establish the background level of signal. In situ data were divided into the following groups: cases with mean grain counts per cell fewer than 5 grains/cell were designated 0, those with 5–10 grains/cell were graded 1, those with 10.1–15 grains/cell were graded 2, and those more than 15 grains/cell were graded 3.

Sections from the same paraffin blocks as were used for ISH were immunostained for Ki-67 nuclear antigen using MIB-1 monoclonal antibody (AMAC, Westbrook, Me). Immunostaining was performed by the avidin–biotin-peroxidase complex method [17]. Briefly, 5-μm thick sections were mounted on silanized slides, dried and stored until ready for use. Sections were brought to water, followed by H_2O_2 pretreatment. Antigen retrieval was performed by microwaving and the use of appropriate retrieval buffer, as previously described [4, 36, 37]. After routine blockage of endogenous peroxidase activity, sections were incubated overnight at 4°C with the MIB-1 monoclonal antibody. Antigen–antibody complexes were detected with the 3,3'-diaminobenzidine/H_2O_2 reaction, rendering MIB-1-immunopositive nuclei dark brown. Slides were counterstained with methyl green, dehydrated, cleared, and mounted.

The nuclei were counted with a 40× objective (×400) without information on the specimens. A minimum of 20 high-power fields was counted per specimen. Both immunopositive and immunonegative nuclei were counted with the aid of a 10 × 10 square grid fitted into the eyepiece of the microscope. Vascular endothelial cells and areas of non-neoplastic adenohypophysis were excluded. In each specimen, the MIB-1 labelling index (LI) was determined and expressed as the percentage of MIB-1-positive nuclei. To evaluate the relationship between IGF-II mRNA expression and MIB-1 LI, the one-way ANOVA, two-pair test using the Bonferroni correction was applied.

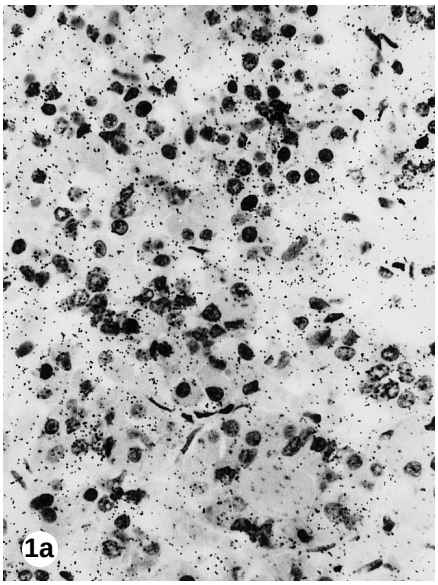
Results

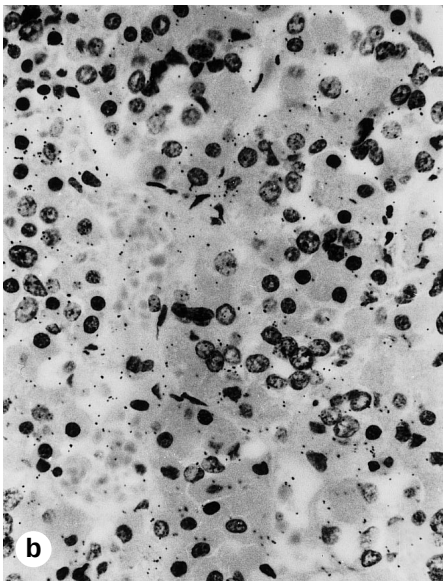
In non neoplastic pituitaries, a positive signal for IGF-II RNA was demonstrated using a higher concentration of probe than in the case of adenomas. In general, the intensity of the hybridization signal did not differ in anterior and posterior lobes. The silver grains were localized in

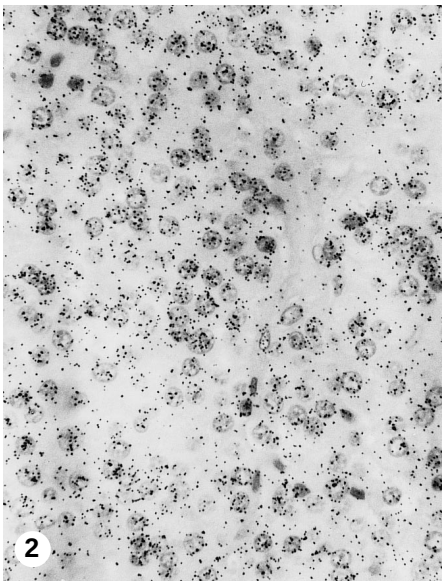
Table 1 The results of in situ hybridization for IGF-II mRNA and immunohistochemistry for MIB-1 in pituitary adenomas

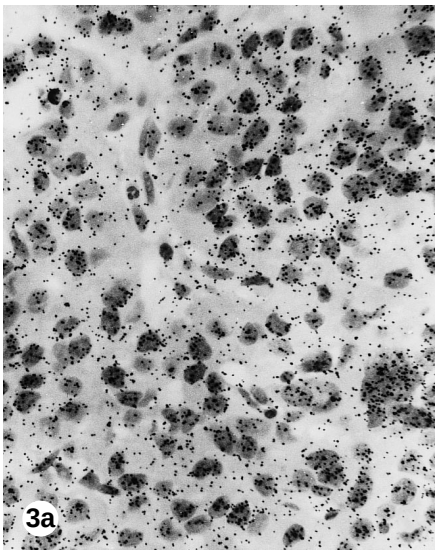
Adenoma type	No. of cases	IGF-II +(No)	Mean IGF-II signal (range)[a]	MIB-1 LI (%)
Densely granulated GH cell adenoma	6	4	1.25 (1–2)	0.73
Sparsely granulated GH cell adenoma	8	7	1.28 (1–2)	0.06
Sparsely granulated PRL cell adenoma	6	0	0	1.09
Mixed GH-PRL cell adenoma	7	6	1	0.51
Mammosomatotrophic adenoma	7	2	1	0.05
Acidophilic stem cell adenoma	5	5	2.2 (2–3)	2.23
Functioning corticotrophic adenoma	6	6	1	0.78
Silent corticotrophic adenoma subtype 1	3	0	0	0.00
Silent corticotrophic adenoma subtype 2	4	0	0	0.38
Silent adenoma subtype 3	8	1	1	0.10
Gonadotrophic adenoma	22	4	1.5 (1–3)	0.41
Thyrotrophic adenoma	6	4	2.5 (2–3)	0.45
Null cell adenoma	7	6	1.33 (1–2)	0.67
Oncocytoma	8	4	1	0.50
Plurihormonal adenoma (GH/PRL/TSH/LH/αSU)	6	6	2 (1–3)	0.07

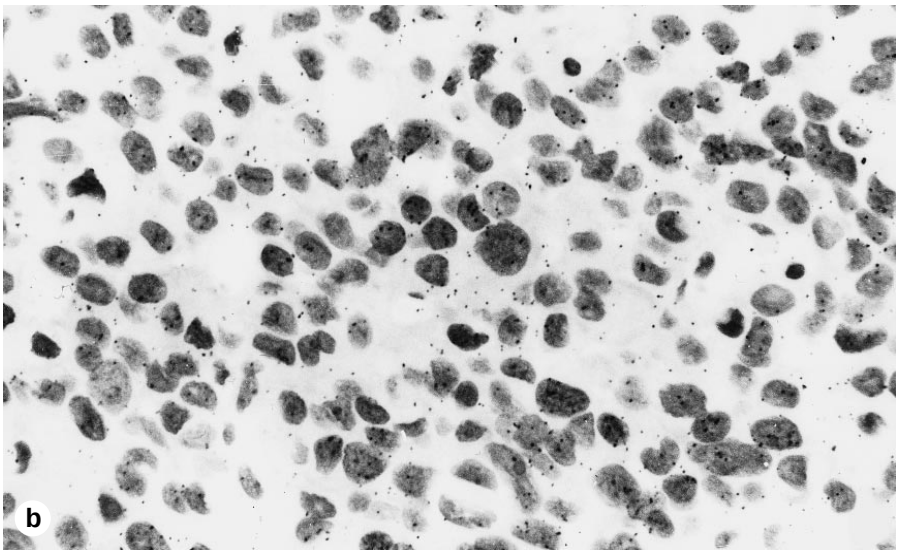
[a] *1* between 5–10 silver grains/cell, *2* 10.1–20 silver grains/cell, *3* >20.1 silver grains/cell











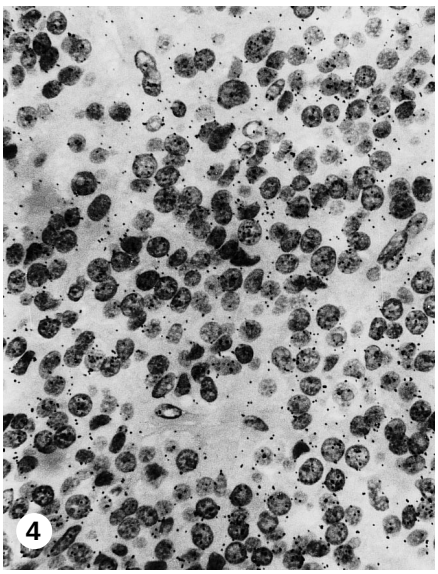


Fig. 1a, b Nontumour pituitary showing positive signal for IGF-II mRNA in most cells. **a** A few cells are negative. **b** The signal is almost abolished by RNase pretreatment. Original magnification ×400

Fig. 2 In situ hybridization for IGF-II mRNA reveals a strong signal in an acidophil stem cell adenoma. Original magnification ×400

Fig. 3 Thyrotrophic adenoma **a** exhibits an intense hybridization signal, which **b** is markedly decreased by comparative study with 100-fold excess of unlabelled probe. Original magnification ×400

Fig. 4 A plurihormonal adenoma shows a moderate hybridization signal for IGF-II. Original magnification ×400

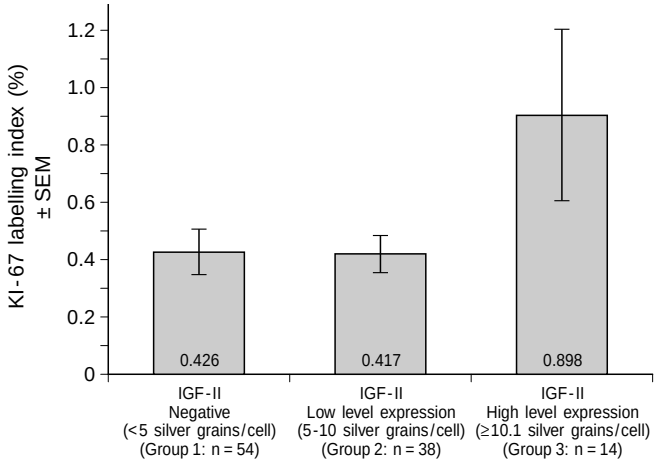


Fig. 5 The MIB-1 labelling index is significantly higher in adenomas with IGF-II RNA signal greater than 10 silver grains/cell than in adenomas without detectable, or with a low level of IGF-II expression; one way ANOVA, F ratio = 3.513, P = 0.33; pairwise comparisons: group 1 vs group 3, P = 0.012; group 2 vs group 3, P = 0.014 (Bonferroni-corrected P-values)

many acidophils, basophils and chromophobes (Fig. 1a). As determined by RNase pretreated controls, silver grain counts of under 5/cell were regarded as background signal (Fig. 1b).

The results of ISH in pituitary adenomas are summarized in Table 1. Fifty-five adenomas (50.4%) were positive, and 54 (49.6%) were negative. Among the GH-producing adenomas, most sparsely granulated GH cell and mixed GH cell–PRL cell adenomas and many densely granulated GH cell adenomas contained IGF-II signal. The hybridization signal was rather weak. All acidophil stem cell adenomas showed a moderate or strong IGF-II signal (Fig. 2). No IGF-II RNA was demonstrated in sparsely granulated PRL cell adenomas. Only corticotrophic adenomas associated with Cushing disease contained IGF-II RNA, while silent subtypes 1 and 2 were negative. Among glycoprotein hormone-producing tumours, a strong or moderate signal was present in most thyrotrophic adenomas (Fig. 3), while some gonadotrophic (both female and male types), null-cell and oncocytic adenomas expressed usually small amounts of IGF-II RNA. In plurihormonal adenomas, the signal varied from weak to intense (Fig. 4a). The hybridization signal was abolished by RNase pretreatment and competition studies with 100-fold excess of unlabelled probe (Fig. 4b).

Immunostaining for MIB-1 was performed, and LI was calculated for 108 pituitary adenomas; in 1 case no more tissue was available for this immunostaining. An average of approximately 1,700 nuclei was evaluated in each specimen. The LI ranged from 0 to 3.20%; the mean value was 0.50 ± 0.06 (mean ± SEM). Acidophil stem cell adenomas had the highest mean LI (1.98%), whereas in silent corticotrophic subtype 1 adenomas no MIB 1 immunoreactive nuclei were found (Table 1).

Two adenomas from patients treated with octreotide up to surgery were excluded from the statistics, since the treatment can affect the percentage of cycling cells. For statistical interpretation, the adenomas with more than 15 silver grains, owing to their small number, were grouped together with the cases with more that 10 silver grains. The mean LI was significantly higher in adenomas with more than 10 silver grains/cell than in the group with up to 10 silver grains/cell or the IGF-II negative group (0.89 vs 0.41 vs 0.42; Fig. 5).

Discussion

The present study shows that IGF-II gene is expressed in many adenohypophysial cells, including the acidophils, basophils, and chromophobes. This is in agreement with the findings in rat anterior pituitary [1]. In contrast to the rodent pituitary, where the posterior and intermediate lobes showed a stronger signal for IGF-II, no such difference was apparent in human pituitary. The diffuse localization of IGF-II mRNA suggests that its role is not restricted to the inhibition of GH production by somatotrophs.

Approximately half the adenohypophysial adenomas contained IGF-II mRNA. They represented all types of tumours, except for lactotrophic and silent subtype-1- and -2 corticotrophic adenomas.

In GH secreting tumours, when present at all the hybridization signal was weak or moderate. Whether the elevated GH blood levels in acromegalic patients influence the expression of IGF-II by adenomas is not known. Postnatally, circulating IGF-II levels remain high in humans and there is with no pubertal increase, whereas in rodents IGF-II levels are very low. Serum IGF-I and IGF-II are reduced by 73% and 52%, respectively in the absence of GH in fetal rat [18]. Protracted GH excess leads to an increase in IGF-II mRNA in rat heart and skeletal muscle, but does not increase the blood or pituitary IGF-II [10, 38]. However, in vitro, IGF-II was shown to inhibit GH release from adult rat pituitary and from human fetal and adult anterior pituitary cells [13, 14], and it is possible that the locally produced IGF-II has an inhibitory effect on adenoma GH production. It has been reported that IGF-II inhibits GHRH-stimulated GH release from cultured rat anterior pituitary cells in a dose-dependent manner [6]. In vitro treatment of GH-secreting human pituitary adenomas with IGF-II had a significant inhibitory effect only in 2 out of 8 cases [15]. The presence of specific membrane receptors for IGF-I and IGF-II was demonstrated in primary cultures of human GH-producing pituitary adenomas [5] and in rat pituitary GH-secreting tumour cell lines [29]. Since all GH-producing adenomas also produce IGF-I, which has a higher affinity for IGF-I R than IGF-II, it may be that IGF-I prevents the effects of IGF-II mediated by the common receptor. We demonstrated the presence of IGF-I R transcripts in all cases examined, representing all types of human pituitary adenomas (unpublished work).

Among ACTH-producing adenomas, a weak IGF-II RNA signal was found only in corticotrophic adenomas associated with Cushing disease. The silent subtypes 1

and 2, were negative; however, owing to the low number of cases examined, the possibility of its presence in these silent adenomas cannot be excluded. The results suggest that IGF-II may play a part in the regulation of ACTH secretion.

In glycoprotein-producing adenomas, the frequency and the intensity of IGF-II signal varied among gonadotrophic, thyrotrophic, null cell, and oncocytic adenomas. Although an in vitro study showed that IGF-II increased basal and GnRH-stimulated LH release by rat pituitary cells [33], its role in the in vivo regulation of gonadotropins is not proven. Thus, mice with disrupted parental *IGF-II* gene are fertile despite their dwarfism [9].

No detectable IGF-II transcripts were present in sparsely granulated lactotrophic adenomas. This is an intriguing finding, since all acidophil stem cell adenomas that secrete prolactin and, less frequently, both prolactin and growth hormone [20, 23] contained IGF-II signal with moderate or strong intensities. These results argue against a role for IGF-II in PRL secretion by tumour cells. This is in contrast to normal pituitary, in which early studies demonstrated an inhibitory effect of IGFs on PRL secretion in vitro [13, 14].

Although the role of IGF-II in hormone secretion by pituitary adenomas has not been clarified these data suggest that it is involved in the stimulation of tumour cell proliferation. This role requires a higher expression of IGF-II gene, as indicated by the significantly greater proliferation index in such tumours than in those with a low level of transcripts. The highest amounts of IGF-II RNA were found in thyrotrophic, acidophil stem cell and plurihormonal adenomas. Among these types, acidophil stem cell adenomas, known for their aggressive behaviour had the highest MIB-1 LI. Thyrotrophic and plurihormonal adenomas can also be aggressive and/or invasive [20] and have a relatively high percentage of cycling cells [19, 21, 37]. However, the groups of thyrotrophic and plurihormonal adenomas in the present study had low MIB-1 LIs. This may be due to the relative small numbers of these rare tumour types examined. It shoud also be borne in mind that MIB-1 is not a perfect marker of proliferation in every case [31, 37].

We shown that IGF-II RNA is present with variable frequency and intensity in most types of morphologically classified human pituitary adenomas. Its greater expression is significantly correlated with MIB-1 derived tumour growth fraction. This finding suggests that IGF-II has a role as stimulator of adenoma cell multiplication. IGF-II involvement in the regulation of hormone production by adenohypophysial tumours has yet to be elucidated.

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