THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigation of Luteinizing hormone-releasing hormone (LH-RH-I) receptor type I and copy number of chromosome 3 and 4 in human uveal melanoma

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DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES DEBRECEN, 2018

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1 Abbreviations

APITD1: apoptosis-inducing, TAF9-like domain 1

BAP1: BRCA1 associated protein-1

BCL-2: B-cell lymphoma 2

B_{max}: maximal binding capacity

BSA: bovine serum albumin

CCND1: cyclin D1

CEP: centromere specific probes

CI: chromosome index

COMS: collaborative ocular melanoma study group

DAB: 3,3'-diaminobenzidine

DAG: diacylglycerol

DAPI: 4',6-diamidino-2-phenylindole

DDEF1: development- and differentiation-enhancing factor 1

DNA: deoxyribonucleic acid

DOX: doxorubicin

EGF: epidermal growth factor

ERK: extracellular signal–regulated kinase

FBS: fetal bovine serum

FISH: fluorescence *in situ* hybridization

FITC: fluorescein isothiocyanate

FSH: follicle-stimulating hormone

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GI: gastrointestinal

GNAQ: guanine nucleotide-binding protein

GnRH: gonadotropin-releasing hormone

GPCR: G protein–coupled receptor

gp100: glycoprotein 100

GRP: gastrin-releasing peptide

HDM2: mouse double minute 2 homolog (MDM2)

HMB-45: human melanoma black, melanoma marker

HPRT1: hypoxanthine phosphoribosyltransferase1

HRP: horseradish peroxidase

IP3: inositol trisphosphate

K_D: dissociation constant

KS test: Kolmogorov–Smirnov normality test

LH: luteinizing hormone

LH-RH: lurteinizing hormone releasing hormone

LH-RH-R-I: type I luteinizing hormone releasing hormone receptor

LTD: largest tumor diameter

LZTS1: leucine zipper tumor suppressor

MEK: MAP2K1 (mitogen-activated protein kinase kinase 1)

MYC: MYC proto-oncogene, BHLH transcription factor

NBS1: Nijmegen breakage syndrome 1 (nibrin)

NMR: nuclear magnetic resonance

OCM: ocular choroidal melanoma

PKC: protein kinase C

PNA: peptid nucleic acid

PVDF: polyvinylidene difluoride

RAF-RAS- MAPK: mitogen-activated protein kinase signal transduction cascade

RNA: ribonucleic acid

RT-qPCR: reverse transcription and quantitative real-time polymerase chain reaction

SDS: sodium dodecyl sulfate

SEM: standard error of measurement

SSC: standard saline citrate

SST: somatostatin

S-100 protein: low-molecular-weight protein

TTT: transpupillary thermotherapy

UM: uveal melanoma

UTR: untranslated region

7TM: seven-transmembrane domain receptors

2 Introduction

Uveal melanoma (UM) is a rare but very agressive tumor: independently of the presently available therapies, the median survival time of these patients is only about 2-8 months and fifty percent of the patients develop metastasis (1). The cause of this malignancy is unknown, but different risk factors have been associated with disease development (oculodermal melanocytosis, light irides, dysplastic naevus syndrome, uveal naevi). UM is more common in Caucasian males (2). In spite of the early diagnosis, UM related mortality rate remained relatively unchanged. Genetic and epigenetic backgrounds of UM are not fully understood and useful prognostic markers for metastasis development have not been well characterized yet (3).

Hypothalamic luteinizing hormone-releasing hormone (LH-RH) is an important primary link making major connection between the brain and the pituitary, playing a crucial role in the regulation of gonadal functions and in vertebrate reproduction (4). The actions of LH-RH and its analogs are mediated by specific, high-affinity G-protein-coupled receptors for LH-RH found on the membranes of the pituitary gonadotrophs and interestingely, in many different human cancer cells (5). Tumoral receptors for LH-RH have been discovered in, prostate, ovarian, pancreatic, endometrial, human breast and colorectal cancers and in non-Hodgkin's lymphomas, human melanomas and renal cell carcinomas. In the last years, a direct receptor-mediated antiproliferative effect of LH-RH analogs on various tumor cells has been considered (6). The receptors for LH-RH on human tumor cells can also serve as targets for LH-RH analogs that can be coupled to different cytotoxic agents (7). In our previous study, we determined that 46 % of UMs express the LH-RH-R-I (8).

Monosomy 3 frequently occurs in UM, approximately 50 % of UM patients show this aberration (9). Gain of 8q (+8q) is found in about 40 % of UM cases. It is common in combination with monosomy 3, and this combination shows a strong relation with metastatic disease (10). Changes in chromosome 6 are present in approximately 40 % of UM. A rearrangement of chromosome 1 is detected in around 25-30 % (11,12). However, chromosome aberrations in UM are obvious, the relationship between their occurrence and prognosis is quite complex and it remains unsolved. The gene encoding LH-RH-R is located

on chromosome 4q21.2, however the numerical alterations of chromosome 4 have never been studied by fluorescence in situ hybridization (FISH) in UM.

In the present thesis, I will present new results about the expression of specific, high affinity LH-RH-I receptors in ocular choroidal melanoma 1 and 3 (OCM-1 and OCM-3) human UM cell lines as useful expreimental models indicating a novel potential molecular target for cancer therapy. Our findings might contribute to the *in vitro* and *in vivo* evaluation of novel therapeutic approaches based on cytotoxic LH-RH analogs or modern powerful antagonistic analogs of LH-RH targeting specific LH-RH-I receptors in UM. Our results clearly demonstrate alterations in chromosome 3 and 4 in human UM. Our results provide new insights into the genetic background of UM. In summary, our genetic data could provide a more precise evaluation of the prognosis of human UM and offer novel therapeutic approaches of the malignancy.

3 Literature review/background

3.1 Uveal melanoma

3.1.1 Definition and prognostic factors of UM

UM is the most commonly primary intraocular malignancy in adults, and is correlated with significant mortality. Melanomas of the uvea are arised from melanocytes. UM may derive from choroid (72 %), ciliary body (23 %) or the iris (5 %) (Figure 1). Choroidal melanomas are the most frequent type of UM and generally display a discoid, mushroom shaped or dome-shaped growth pattern (1). The estimated incidence is 6-7 cases per one million subjects yearly in the recent decades (2). Over the past decades, the incidence has remained stable, unlike trends indicating a higher incidence of cutaneous melanoma. Although different case control and epidemiologic studies have been evaluated to examine the influence of sunlight exposure in the development of UM, the results are controversal and not conclusive (13,14). Several histologic prognostic factors have been described for UM, such as onset location, the LTD, age at the time of the diagnosis, involvement of the ciliary body and presence of epitheloid cells (15). Several risk factors have been associated with the development of the disease such as uveal naevi, light irides, oculodermal melanocytosis and dysplastic naevus syndrome, however, how and why UM exactly develops, is still largely unknown (16). Oculodermal and ocular melanocytosis are about 35 to 70 times more common in UM patients (17). Well-known clinical prognostic factors are age since the elderly patients tend to have a worse prognosis. Patients with larger tumors and in those patients who have developed metastasis or tumors that ruptured through the Bruch's membrane, UM tumors were significantly more often located anterior to the equator (18). The presence of tyrosinase transcripts in the peripheral blood, suggesting circulating melanoma cells is correlated with the disease stage and predicts progression of disease in patients with early as well as advanced melanoma (19). Nearly 50 % of the patients suffering from UM develop metastatic disease that mostly relates the liver and is almost inescapable lethal. When metastases develop, the median survival of the patients is only 5-7 months (20,21).

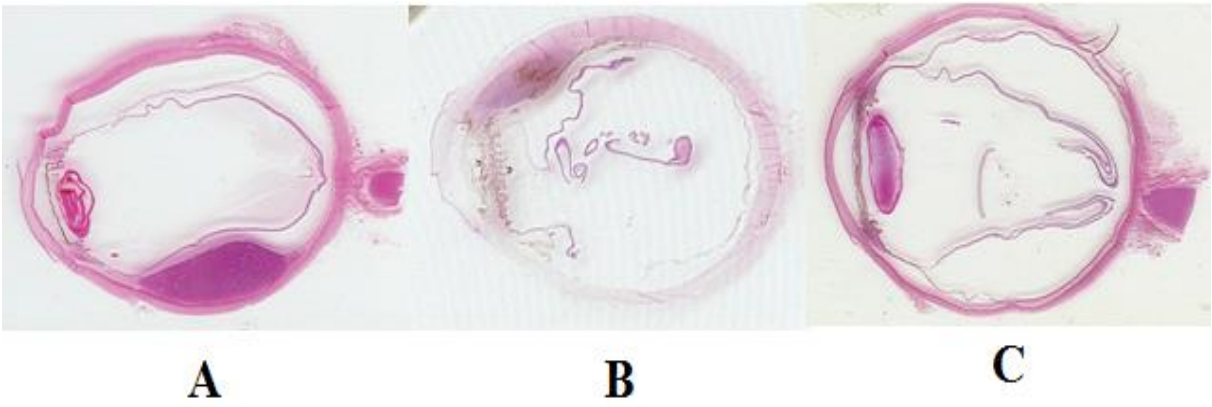

Figure 1: Uveal melanoma located in choroid A, ciliary body B and iris C.
(van den Bosch T, Kilic E, Paridaens D, et al.: Genetics of uveal melanoma and cutaneous melanoma: two of a kind? Dermatol Res Pract 2010: 360136, 2010. Modified (22)*)*

3.1.2 Diagnostics of UM

About 30 % of the patients do not have any symptoms at the time of diagnosis but if there are any complaints the following occour most often: blurred vision, floaters, visual field loss and photopsias (23).

Clinically, the primary diagnosis of UM in the choroidea, often involves decreased visual acuity and scotoma secondary to retinal detachment, with slit lamp biomicroscopy showing melanotic or amelanotic tumors with or without orange dusting. The examiniation is often supported showing acoustic hollowness by an ultrasound investigation (24). Iris melanomas are readily detectable by slit lamp biomicroscopy, whereas ciliary body tumors are covert behind the iris and can be visualized by ultrasonography. Choroidal types of tumor are diagnosed by indirect ophthalmoscopy and ultrasonography. (18).

Diagnostically, small melanomas need to be differentiated from benign nevi. The presence of orange pigment, subretinal fluid and the documented growth on fundus photography are findings that help the diagnosis of melanoma. Ocular echography and fluorescein angiography are the most effective diagnostic tools available for physicians. In some cases, a diagnostic biopsy may be indicated and fine-needle aspiration has to be performed (25).

3.1.3 Treatment of UM

The most important clinical prognostic factor is the size of the tumor, moreover, the therapy is often chosen based on tumorsize. UM are subdivided into different groups depending on the apical size and diameter, however, many centers use their own definition. According to COMS study, the most widely used definition is: "Small melanomas are 1.0 - 2.5 mm in apical height and < 5.0 mm in the largest basal dimension. Medium tumors are defined as tumors between 2.5 and 10 mm in apical height and ≤ 16 mm in the largest basal diameter. Large tumors are ≥ 2 mm in apical height and > 16 mm in maximal basal diameter, or a melanoma > 10 mm in apical height, regardless of the basal diameter" (Collaborative Ocular Melanoma Study Group, 2003) (18).

Different eye-conserving treatment modalities have been ameliorated such as Iodine-125 (I-125) or Ruthenium-106 (Ru-106) plaque brachytherapy, stereotactic radiotherapy, proton beam radiotherapy, phototherapy (photocoagulation or TTT) and transscleral or transretinal local resection (23). Primary tumors are treated by brachytherapy using radioactive plaques to preserve the tissues of the eye (26). Enucleation (removal of the eye) remains a possible therapy for very large tumors and in patients for whom radiotherapy is probable to be problematic. The presence of epithelioid cell type and microvascular loops is correlated with a worse prognosis (25). UM is highly resisting to systemic cytotoxic chemotherapy (23). About 90 % of the patients with metastatic disease have hepatic involvement, other sites including the skin, bones, lung and the brain (23). Treatment by systemic or intrahepatic chemotherapy or partial hepatectomy only rarely prolongs life (27).

3.1.4 Histopathology of UM

Tumor cell type is an important prognostic factor. Three histopathological UM categories are being characterized: spindle, epithelioid and mixed cells (28) (Figure 2). Epithelioid cells have abundant glassy cytoplasm, a well-defined border, and plentiful extracellular space between cells. Nucleoli are very large and eosinophilic within the center of the nucleus. Spindle cells are smaller, less pleomorphic, with smaller nuclei, and stacked tightly with little extracellular space (29). Based on their nucleus, spindle cells can be subcategorized. Subtype A has a narrow nucleus with fine chromatin and indistinguishable nucleolus. Subtype B has a rounder nucleus, thicker chromatin, and more prominent nucleoli (29). The mixed-cell type melanoma has various rate of epithelioid and spindle cells with a minimum of 10 % of any one type (18).

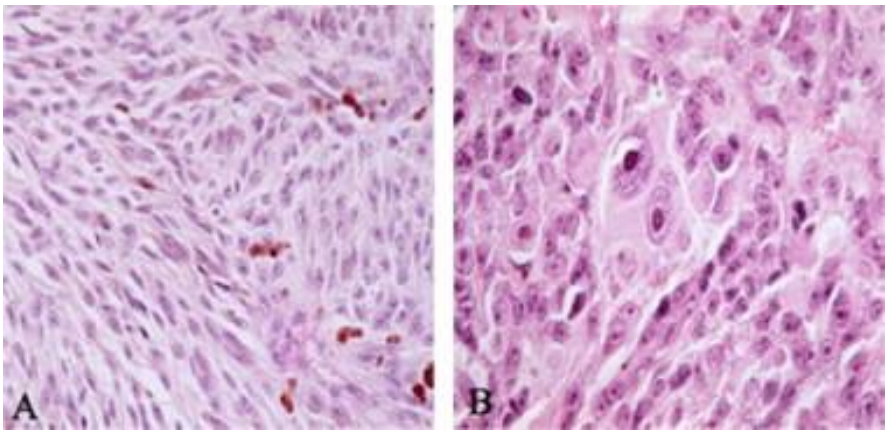

Figure 2: Histopathology of uveal melanoma: (A) spindle cells and (B) epithelioid cells *(Miyamoto C, Balazsi M, Bakalian S, Fernandes BF and Burnier MN: 26: 145–149, 2012*. (29)*).*

Immunohistochemistry can be of diagnostic value. S-100 is detected by cells of neuroectodermal origin. HMB-45 connects to gp100, an antigen expressed by melanocytes that may be useful in differentiating UM from nonmelanocytic tumors (30).

3.2 Genetics of UM

3.2.1 Aberrations of chromosome 3

Loss of one copy of chromosome 3 strongly correlates with metastatic risk and other chromosomal alterations also associate with metastatic diseases (21,31). Most frequently in the liver, 50 % of the patients develop metastases. Monosomy 3 correlates with epitheloid type of tumor, poor outcome and ciliary body involvement (23,32). Lack of chromosome 3 has been investigated in 5-10 % of all the patients. If the remaining chromosome is duplicated, this isth isodisomic state of chromosome 3 and it is prognostically equal to monosomy 3 (33). Infrequently, partial deletions of chromosome 3 have been investigated. A common region of allelic loss on 3p25 and on 3q24–q26 could be observed. Most likely these regions harbored putative tumor suppressor genes, but no specific genes have yet been characterized (34). UM can be divided into 2 groups based on the status of chromosome 3: class 1 tumors with 2 chromosome, and a class 2 tumors with loss of one copy of

chromosome 3 (35). Class 2 tumors have a higher chance of aneuploidy and patients have a high risk to develop metastases whereas class 1 tumors demonstate low aneuploidy and patients rarely have metastases (36).

3.2.2 Aberrations of chromosome 8

Gain of 8q (+8q) is investigated in about fourty percent of UM patients and proved to be an independent significant prognostic marker for decreased survival (37). It has also been characterized in combination with monosomy 3, either as +8q or as isodisomy 8q, and this combination also predicts a strong correlation with metastatic disease (37). The common region of amplification was detected to range from 8q24.1 to 8q24.3. Interestingely, *LZTS1,* is a potential metastasis suppressor gene harbored in 8p21 (38).

3.2.3 Other chromosomal aberrations in UM

In UM, other frequent chromosome aberration, such as loss of 1p and 16q, have been detected. One of the proposed tumor suppressor genes, *APITD1,* in the 1p36 region was shown to be negligable for survival-rate and the common deleted regions on chromosome 1 were detected to range from 1p34.3 to 36.2 (11) Alterations of chromosome 6 are frequently investigated in UM. The region of common deletion was found to range from 6q16.1 to 22.3 on the long arm (39). Moreover, alterations of the other chromosomes such as loss of 9p, loss of chromosome 10, loss of 11q23– q25, and gain of chromosomes 7 and 10 have been detected (34).

3.2.4 Candidate genes in UM

Potential oncogenes, *MYC* is expressed in around 30 % of the UMs (40). *DDEF1* and *NBS1*, other oncogenes on chromosome 8q have been described. (36).

Leading to excessive cell proliferation, the RAS-RAF-MEK-ERK pathway or MAPK pathway is activated in a large proportion of the UMs (41). GNAQ mutations have been found in 50 % of the UM patients. *GNAQ* is a heterotrimeric GTP-binding protein α subunit that binds G-protein coupled receptor signaling to the RAF/MEK/ERF (42).

BAP1 is located on chromosome 3p21.1 and is thought to be a potential tumor suppressor gene. Inactivating somatic mutations have been found in 84 % of the metastasizing UMs, implicating that *BAP1* mutations seems late in the UM progression (43).

3.3 Peptide hormon receptors as molecular targets in cancer therapy

The discovery of specific, high affinity receptors for several hypothalamic hormones on various human cancer cells has led to the successful development of novel radiolabeled and cytotoxic hormone analogs. These new peptide analogs are more selective in wiping out human cancer cells and less toxic than conventional chemotherapeutic agents (4,44–46).
Among others, LH-RH, somatostatin, bombesin/GRP, their mRNAs, and their receptors are found in various tumors (4,45,46).

3.3.1 Somatostatin and somatostatin receptors

SST is a hormonal neuropeptide that occurs in several active forms: SST14 (which consists of 14 amino acids) and SST28 (an N-terminally elongated variant) (45,47). Both forms have antiproliferative activity and inhibit the secretion of many hormones (4,44) SSTs can bind to five mammalian SST receptors: sst1, sst2A/B, sst3, sst4 and sst5 (47). SST-14 has short half-life. More stable SST analogs such as octreotide, vapreotide and lanreotide have been developed for human therapy. (4,44). SST and its analogs show direct and indirect antitumor effects (48,49). They directly inhibit tumor growth and can interact with specific membrane receptors on tumor cells (48). SST and its analogs can also indirectly affect metastasis by inhibition of angiogenesis, since tumor angiogenesis is needed for tumor growth, invasion and metastasis (48,49). The localization of tumors and metastases can be visualized by scintigraphic techniques (50). [^{111}In-(DTPA)-$_{D}$-Phe^{1}]-octreotide (OctreoScan) are widely used for visualization of SSTR-expressing tumors (50). Targeted radiotherapy, in which SST analogs are linked to various radio- nuclides such as ^{90}Y or ^{68}Ga, is also being investigated (44–46). SST receptor subtypes are known to be expressed in human breast cancer, ovarian, endometrial, prostate, colorectal cancer and renal cell carcinomas (51–56). The receptors for SST on human tumors might also serve as targets for SST analogs linked to cytotoxic agents. A highly active targeted cytotoxic SST analog AN-238 consists of AN-201 linked to an octapeptide carrier RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2). AN-238 inhibits the growth of experimental breast, ovarian, pancreatic, prostate, renal and lung cancers, as well as brain tumors and their metastases that express SSTR-2 or -5 (44,45). It can be used to the development of therapeutic approaches targeting SST receptors.

3.3.2 Bombesin/GRP and bombesin/GRP receptors

Bombesin-like peptides are not classical hypothalamic hormones since they play only a perfunctory role in the release of pituitary hormones. They are present in the mammalian brain, including the hypothalamus as well as in lung and GI tract (4,45,46). The most important oncological effect of bombesin/GRP is playing a crucial role in the growth and/or differentiation of various human tumors including breast, prostate and pancreatic cancer (57–59). Four receptor subtypes for the bombesin/GRP have been described (57,58). GRP receptors can influence cell cycle progression from G1 to S-phase, induce activation of tyrosine kinases, and lead to EGF receptor transactivation (57,60). Bombesin/GRP receptors are overexpressed in various human malignancies including prostate, breast, pancreatic, colon, gastric cancer, neuroblastomas and brain tumors (4,45,57,61,62). Numerous radiolabeled GRP analogs have been developed (57). Clinical studies with [^{99m}Tc]- and [^{68}Ga]-labeled bombesin-based peptides have been reported for imaging metastasized prostate, breast and gastrointestinal tumors (45,57). Cytotoxic bombesin conjugates using bombesin/GRP antagonists as carriers have been synthesized (45,63). The cytotoxic bombesin analog AN-215 was prepared by linking the amino terminal of des-D- Tpi-RC-3095 through a glutaric acid spacer to the 14-OH group of 2-pyrrolino-DOX (AN-201) (4,45,63). The proliferation of xenografts of small cell lung cancer as well as in prostate, renal, mammary, ovarian, endometrial, pancreatic and gastric cancers and brain tumors in nude mice was strongly inhibited by AN-215 (44,46,63–65). Cytotoxic analogs of bombesin /GRP are still to be tested clinically.

3.3.3 Luteinizing hormone-releasing hormone type I (LH-RH-I) and LH-RH-I receptors

Hypothalamic luteinizing hormone-releasing hormone type I (LH-RH-I) also known as Gonadotropin-releasing hormone (GnRH) is the primary link between the pituitary gland and the hypothalamus in the regulation of gonadal functions and it has a determinant role in vertebrate reproduction. The actions of LH-RH-I are mediated present on the plasma membranes of the pituitary gonadotrophs by specific G protein-coupled receptors for LH-RH-I (4,45). The amino acid sequence of LH-RH-I (pyroGlu-His-Trp-Ser-Tyr-Gly- Leu-Arg-Pro-Gly-NH_2) was reported nearly at the same time by the research groups of Schally and

Guillemin, independently from each other. For this discovery they received the Nobel Prize in medicine or physiology in 1977.

LH-RH-I activates the Gqa protein leading to the formation of IP3. This activation stimulates intracellular Ca^{+2} release in steps necessary for exocytosis of FSH and LH secretory granules (66). The amino-terminal residues of LH-RH-I play important role in receptor activation, and alteration of these residues in LH-RH-I produces analogs with antagonistic properties (67) (Figure 3, 4)

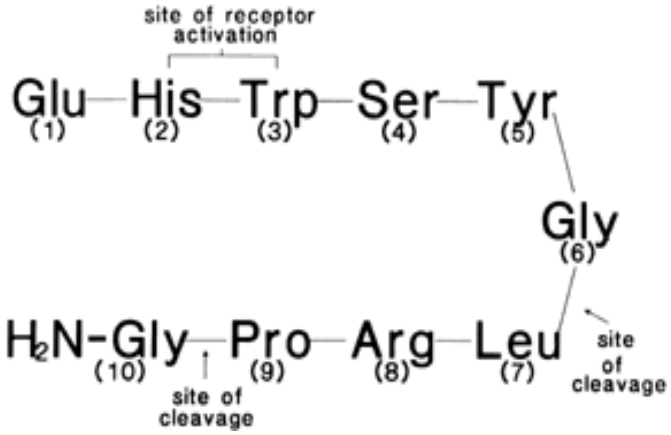

Figure 3: Chemical structure of LH-RH-I. The molecule is bent around the flexible glycine in position 6. Amino acids 2 and 3 are important for receptor activation. (*https://www.glowm.com/resources/glowm/cd/pages/v5/ch059/framesets/004f.html*)

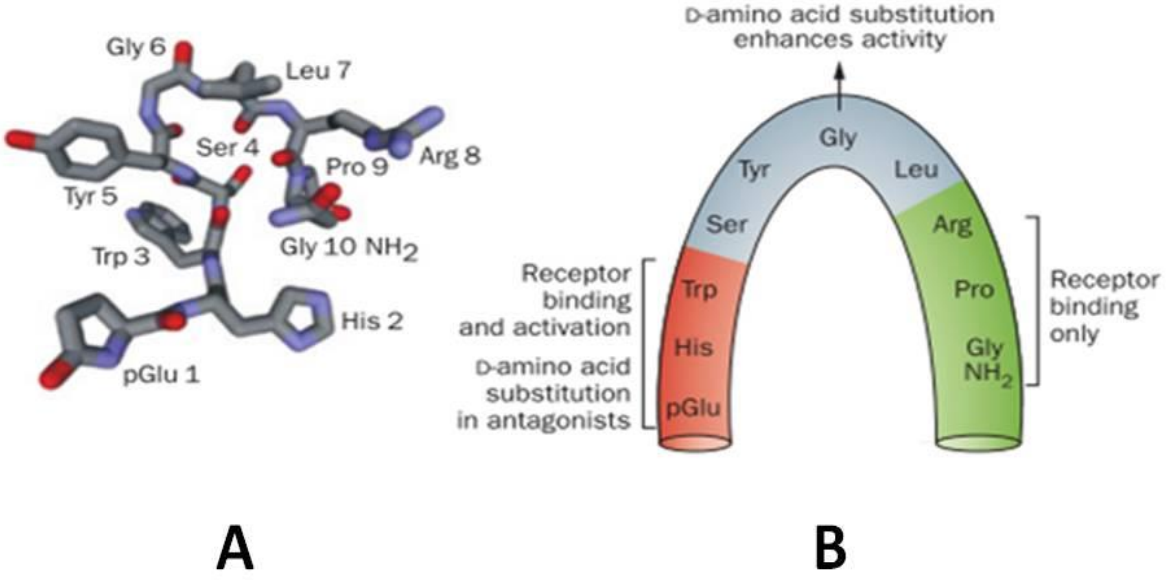

Figure 4: The 3D structure of LH-RH-I A: NMR structure of LH-RH-I B. Schematic representation of LH-RH-I in the folded conformation *(Millar RP and Newton CL: Current and future applications of GnRH, kisspeptin and neurokinin B analogues. Nat Rev Endocrinol 9: 451–66, 2013* (68)*)*

Several LH-RH analogs substituted in positions six, ten, or both are much more active and possess prolonged activity than LH-RH without any substitutions (69). The most important analogs are: Triptorelin; leuprolide, buserelin, goserelin and nafarelin, which are 50–100 times more potent than LH-RH (69). Paradoxically, chronic administration results in inhibitory effects due to receptor desensitization, although an acute injection of superactive agonists of LH-RH produces a significant release of LH and FSH (70). LH-RH analogs have direct inhibitory effects on prostate, breast, endometrialand ovarian cancers mediated through specific LH-RH receptors on the tumor cells (71)

There are several isoforms of LH-RH. The human LH-RH-I gene is located as a single gene copy on chromosome 8p11.2-p21 and is consisted of 4 exons separated by 3 introns. The human LH-RH-II gene has been located to chromosome 20p13. The human LH-RH-II gene (2.1 kb) is shorter than the LH-RH-I gene (5 kb) (72). The most significant difference in tissue distribution of LH-RH-I and LH-RH-II in humans is that the LH-RH-II is expressed at

the highest level outside the brain (72). Interestingly, both forms of LH-RH are overexpressed in breast cancer and are expressed in normal human breast tissue (73).

The human LH-RH type I receptor is a member of the GPCR superfamily and has 328 amino acids. It differs from most other seven transmembrane, GPCR by lacking a C-terminal, cytoplasmic tail. The gene encoding for the type I LH-RH receptor located on chromosome 4q21.2 and composes of 3 exons divided by 2 introns (74) (Figure 5).

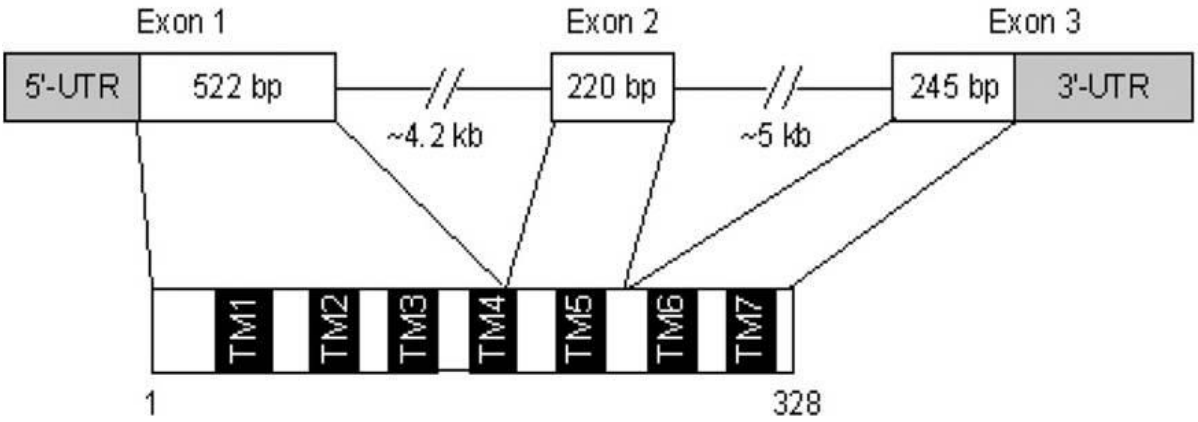

Figure 5: Human type I LH-RH receptor gene. Exon 1 contains the 5'-UTR and encodes the first three TM domains and a portion of the fourth TM domain. Exon 2 is 220 bp in length and encodes the remainder of the fourth TM domain, the fifth TM domain, and part of the third intracellular loop. Exon 3 encodes the rest of the open reading frame and contains the 3'-UTR *(Cheng CK and Leung PCK: Molecular biology of gonadotropin-releasing hormone (GnRH)-I, GnRH-II, and their receptors in humans. Endocr Rev 26: 283–306, 2005* (74) *Modified)*

A major substance of receptor function is binding of the ligand, and this interrelationship is the primary determinant of whether a receptor starts signaling within the cell (75).
The LH-RH-R-I is connected to Gq/11 proteins to activate phospholipase C which transmits its signal to DAG and IP3 (Figure 6). IP3 stimulates release of intracellular calcium and DAG activates the intracellular PKC pathway (76). PKC activation in response to LH-RH also leads to increases in the MAPK in pituitary cells. The active MAPKs proceed to the nucleus where they activate different transcription factors to regulate gene expression. These signaling

pathways then differentially modulate the synthesis and secretion of the gonadotropin sub-units and selectively modulate gonadotropin synthesis and/or release from pituitary cells (76).

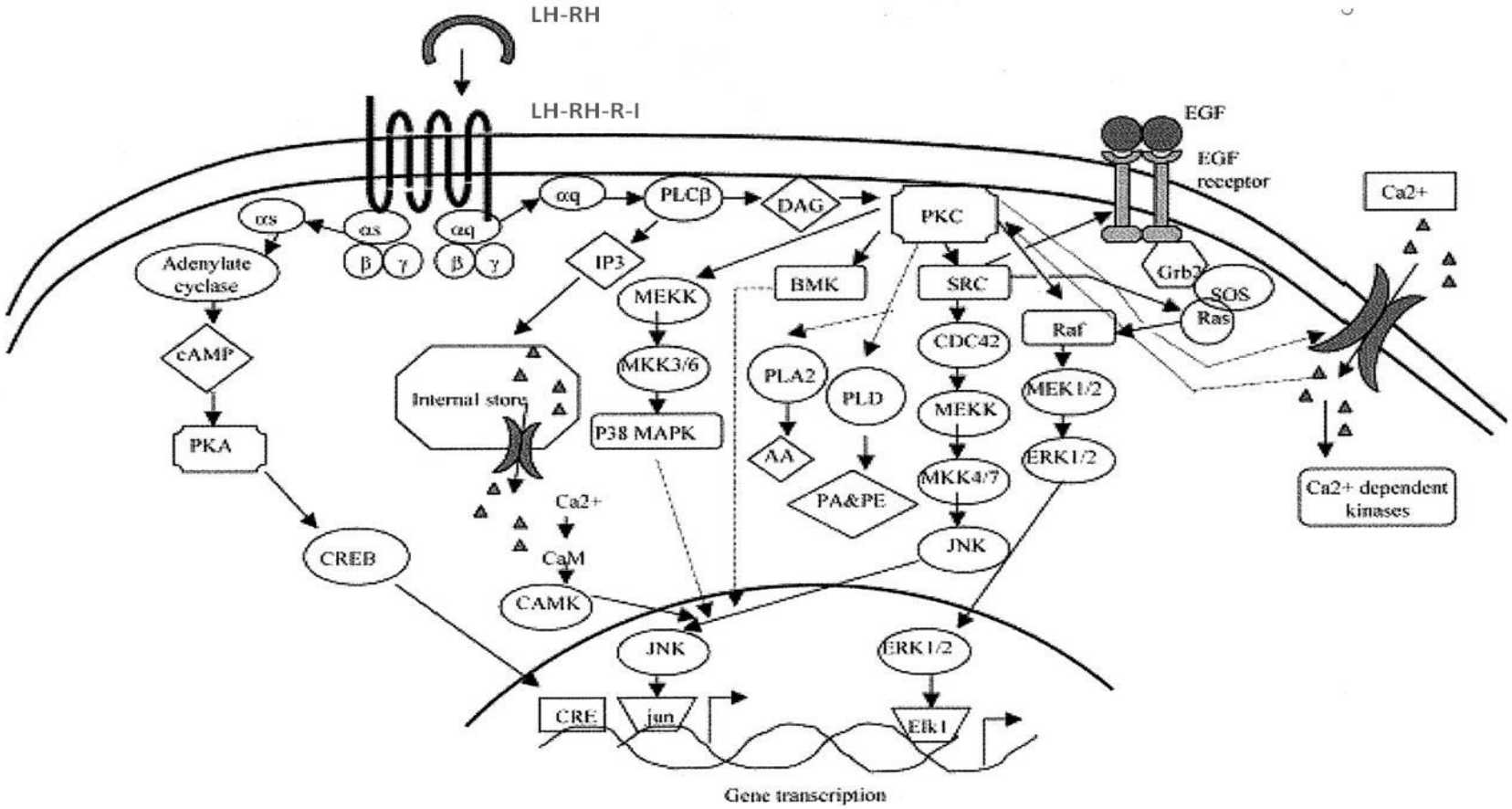

Figure 6: LH-RH type I receptor activation and singaling pathway *(Harrison GS, Wierman ME, Nett TM and Glode LM: Gonadotropin-releasing hormone and its receptor in normal and malignant cells. Endocr Relat Cancer 11: 725–748, 2004* (77) *modified*

The presence of different chemical forms of LH-RH is deeply connected with the existence of various LH-RH receptor subtypes. Based on previous research in vertebrates, three subtypes of specific LH-RH receptors have been identified and characterized. Principally, type I receptors are located in the pituitary and mediate the regulation of gonadotropin secretion. LH-RH-R-I is mainly localized in the hypothalamus, however, it has also been detected in the breasts, endometrium, placenta, ovary, testis, prostate, kidneys, thymus and in lower levels in various other organs (4,45,46,69,78)

Tumoral receptors for LH-RH have been developed on human breast, ovarian, endometrial, prostate, pancreatic and colorectal cancers, renal cell carcinomas, human melanomas, and non-Hodgkin's lymphomas (45,46,78–80). Previously, we have also demonstrated the expression of LH-RH type I receptors and LH-RH ligand in human UM specimens (8). The receptors for LH-RH on human tumors might also serve as targets for LH-RH analogs coupled to cytotoxic agents (4,44,45,81,82). The cytotoxic analog, AN-152 (AEZS-108), widely used in targeted therapy, consists of DOX covalently linked to [D-Lys^6]LH-RH, an LH-RH agonist (Figure 7) (7). AN-152 binds specifically to LH-RH receptors through its peptide moiety, after internalization releases in the lysosomes and induces apoptosis (7,83). AN-152 has been tested in phase II and III clinical trials in human ovarian and endometrial cancers and in phase I/II trials in castration resistant human prostate cancer (7,82,84,85). In some cancers, locally produced LH-RH support the evidence for an autocrine and/or paracrine regulatory system with LH-RH receptors (8).

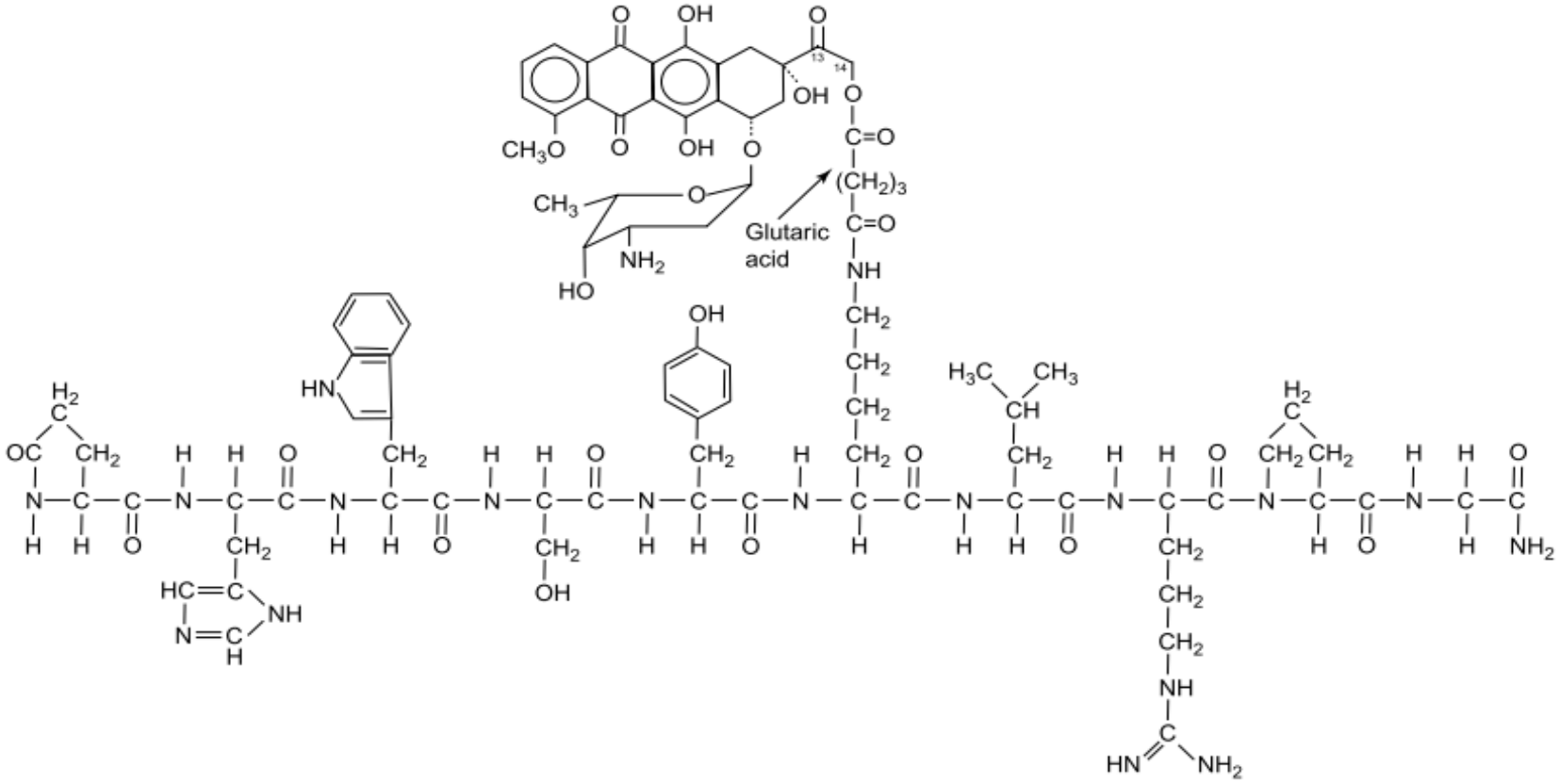

Figure 7: Molecular structure of cytotoxic luteinizing hormone-releasing hormone (LH-RH) analog AN-152. (*Schally A V. and Nagy A: Chemotherapy targeted to cancers through tumoral hormone receptors. Trends Endocrinol Metab 15: 300–310, 2004* (46)*)*

3.4 The role of fluorescence *in situ* hybridization in UM

Fluorescence *in situ* hybridization (FISH) implies the preparation of short sequences of single-stranded DNA which are complementary to the DNA sequences. These probes connect to the complementary DNA. The probes are labeled with fluorescent tags and allow to see the location of those sequences of the complementary DNA (Figure 8.) (86).

FISH can detect chromosomal alterations that are consequent with a diagnosis of neoplasia. Different studies have shown that FISH has significantly higher sensitivity for the detection of tumor cells than conventional cytology (87–89). FISH is also able to identify various types of cytogenetics alterations including duplication, aneusomy, deletion, amplification and translocation (90). Today, genetic alterations by FISH can be detected within 24 hours. FISH is now part of the routine diagnostics. It plays an important role in planning therapies, and monitoring diseases. It can be used to visualize the organization of chromatin structure, to identify genetic disorders caused by radioactivity, to detect genetic disease and chromosomal abnormality (86).

FISH probes may be DNA or RNA molecules. There are also peptid PNA probes, where a synthetized peptidchain replaces a sugar-phosphate backbone. Usually, three basic types of DNA probes are used: whole chromosome (whole chromosome paints), centromeric (chromosome enumeration probes) and locus-specific probes (86).

FISH was used first in UM studies by Sisley in 1997 (91) Since this publication FISH has been widely used to study UM cytogenetics, with monosomy 3 occurring at a rate of 30%-50% in most samples (92). It has the potential to be a useful tool for detecting chromosomal changes for prognostic purposes in UM (92).

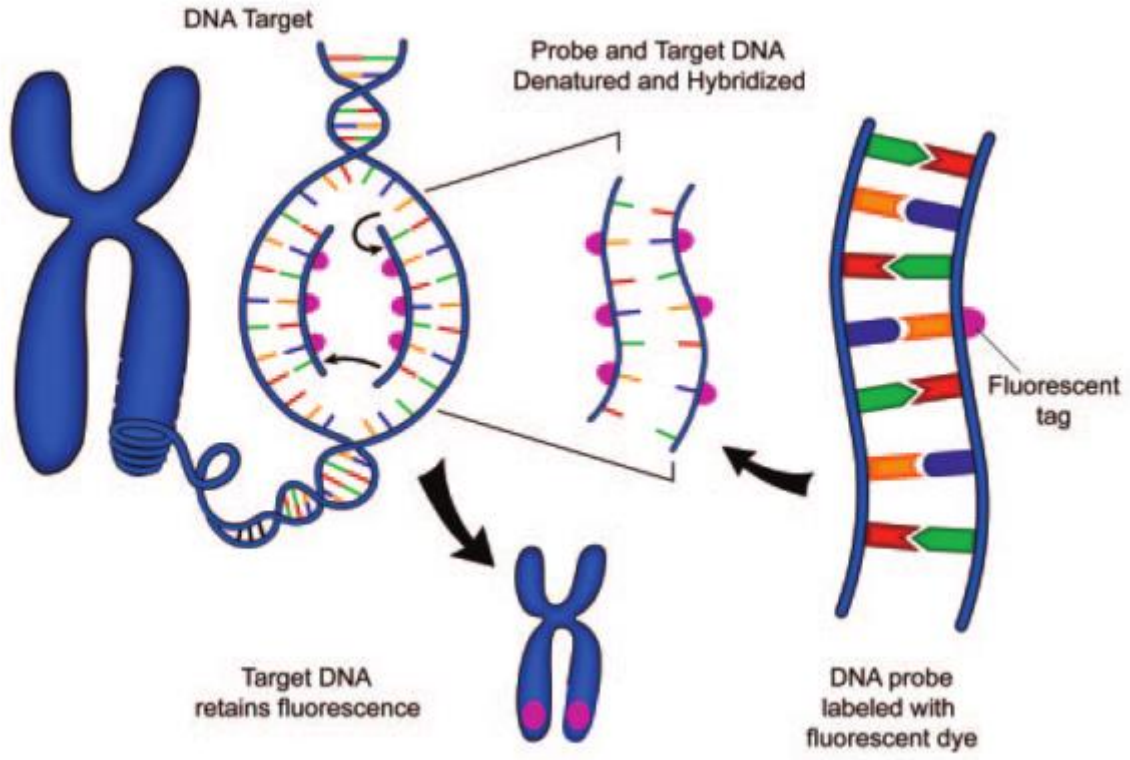

Figure 8: Schematic representation of FISH technique. A DNA probe is tagged with a fluorescent marker. The probe and target DNA are denatured, and the probe is allowed to hybridize with the target. The fluorescent tag is then detected with a fluorescent microscope. *(Wippold FJ and Perry A: Neuropathology for the neuroradiologist: Fluorescence in situ hybridization. Am J Neuroradiol 28: 406–410, 2007* (93)*)*

4 Aims of the study

Previously, we have demonstrated the expression of LH-RH ligand and specific, high affinity LH-RH type I receptors in human UM specimens (8). The gene encoding LH-RH-R-I is harbored by chromosome 4q21.2, however the numerical alterations of chromosome 4 have never been examined by FISH in UM.

In this study, our aim was:

1. Examine the mRNA expression of LH-RH ligand and LH-RH-I receptor in OCM-1 and OCM-3 human UM cell lines as useful models for further *in vitro* and *in vivo* studies.
2. Determine the existence and binding characteristics of LH-RH-I receptor protein by Western blotting, immunocytochemistry and ligand competition assays.
3. Investigate the expression of mRNA and protein of LH-RH-I receptors in tumor cancer samples from nude mice xenografted with OCM-1 and OCM-3 cell lines.
4. Determine the copy number of chromosome 3, particularly the monosomy of chromosome 3 which has been substantially described in the aggressive behavior of UM, and chromosome 4 in 46 human UM specimens using FISH.
5. Investigate chromosome index (CI) and „dominant" cell population values for chromosome 3 and 4.
6. Examine the survival rate of the UM patients according to their CI.
7. Determine the correlation between LH-RH and LH-RH-R-I expression and the copy number of chromosome 3 and 4.

5 Materials and methods

5.1 Cell lines and culturing

OCM-1 and OCM-3 human primary UM cell lines were cultured in RPMI 1640 medium supplemented with L-glutamine, 10 % FBS, and 1 % penicillin/streptomycin in a humidified chamber in 5 % CO_2 at 37°C. Cells were subcultured every 3 days using a standard trypsinization procedure.

5.2 Animal studies

Athymic (nude) mice (Ncr nu/nu) were obtained from Charles River Laboratories (Germany). Mice were housed in sterile, individually ventillated cages in an air-conditioned (21±2 ºC), humidity-controlled room (≈50 %) with a 12/12 hour light/dark cycle. Animals were fed with autoclaved chow and water *ad libitum*. All experiments were in accordance with the institutional guidelines for the welfare of experimental animals and regulations of the European Union. The experimental protocol was approved by the Laboratory Animal Care and Use Committee of the University of Debrecen. Six million tumor cells were subcutaneously injected into the femoral region of the mice. Four weeks after the initiation of donor animals, when tumors had developed in donor animals, tumors were aseptically dissected and mechanically minced. Approximately 3 mm^3 tumor tissue was transplanted subcutaneously into nude mice by a trocar needle. At the end of each experiment, mice were sacrificed under 3 % isoflurane anaesthesia using a small animal anaesthetic device. Tumors were excised, weighed and necropsy was done. Tumor specimens were snap-frozen and stored at - 80°C until further experiments.

The generation of the experimental animals was carried out with the help of one of my co-authors, David Rozsa.

5.3 Human UM tissues

Specimens of human UM were obtained from 46 patients 30-84 years of age at the time of enucleation at the Department of Ophthalmology of the University of Debrecen, Hungary

from 2008 to 2013 (Figure 9.). Normal lymphocyte samples, used as positive controls were collected at the Department of Pathology of the University of Debrecen. Informed consent was obtained before enucleation, and the study was performed according to the tenets of the Declaration of Helsinki and the local Institutional Ethics Committee. Fresh tumor tissue was obtained less than one hour after enucleation, according to a standardized protocol. Briefly, an incision was made through the tumor, leaving the optic nerve intact. The quantity of tissue obtained (5-8 mm^3) based on the size of the tumor. Sample was taken from the side opposite the optic nerve and selected portions of the melanoma tissues were flash frozen and stored at -80°C. Conventional histopathological examination was performed on all tumors and the origin of the tumor was confirmed. Follow-up data from the time of diagnosis until the end of the study were obtained by reviewing the medical records of the patients (if we had the availability) and/or contacting their general physicians. The clinicopathological data of the 46 patients are summarized in Table I. UM samples were divided into 4 groups, based on the CI: NN (normal CI3 and CI4), NP (normal CI3 pathological CI4), PN (pathological CI3 and normal CI4) and PP (pathological CI3 and CI4). To simplify the evaluation, two major groups were also created: N (including NN) and P (containing NP, PN and PP).

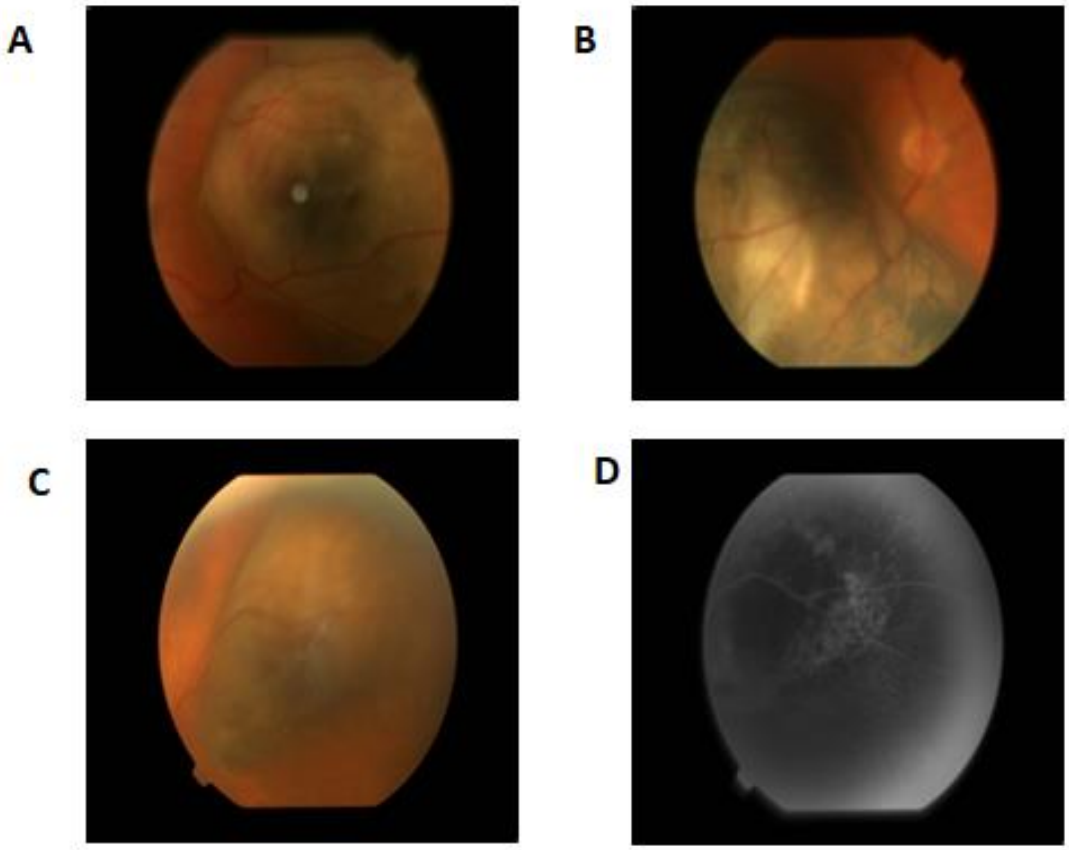

Figure 9. A: Choroidal melanoma in the left eye (Patient No. 13) B: Choroidal melanoma in the right eye (Patient No. 26) C, D: Fundus and fluorescein-angiographic image of choroidal melanoma in the left eye (Patient No. 17)

Table I. Clinicopathological characteristics, CI results and survival data of 46 UM patients

Sample ID	Sex	Age	Type	Eye	Localization	Survival	CI3	CI4	Postoperative Days
1	f	79	ND	l	C	dead	1.43	2.72	**210**
2	m	76	spindle	l	P	alive	2.00	2.65	**1559**
3	f	44	spindle-B	l	inferior temporal: P	alive	2.19	3.39	**1770**
4	f	50	spindle	r	temporal: P	alive	2.41	3.00	**1497**
5	m	76	spindle	r	P	dead (liver)	2.18	3.94	**620**
6	f	30	spindle-A	l	P	alive	2.17	3.34	**1770**
7	m	66	epithelioid	l	temporal: P	alive	2.04	4.01	**333**
8	m	61	spindle-B	l	temporal: P	alive	2.21	2.81	**1505**
9	m	53	ND	l	superior temporal: P	alive	2.04	3.94	**1260**
10	m	53	epithelioid	r	P	alive	1.48	2.79	**1442**
11	f	79	epithelioid	r	P	dead	2.07	3.43	**548**
12	m	67	epithelioid	l	P	alive	2.10	2.53	**1630**
13	f	72	epithelioid	l	temporal: P	dead (liver)	1.37	5.39	**317**
14	m	35	spindle	l	superior nasal: P	alive	1.71	2.94	**740**
15	m	55	spindle-B	l	P	alive	2.68	3.03	**1545**
16	m	65	spindle-B	r	anterior temporal: P	dead	2.53	1.91	**467**
17	f	68	spindle	l	P	alive	2.07	1.75	**1702**

18	m	71	spindle-B	r	P	alive	2.28	3.43	**1006**
19	m	69	mixed	r	anterior nasal: P	alive	1.37	2.31	**958**
20	m	64	ND	l	temporal: P	dead (bone)	1.79	2.39	**312**
21	f	75	epithelioid	l	temporal: P	alive	2.26	3.04	**846**
22	f	79	ND	r	C	alive	2.43	2.36	**1442**
23	f	75	mixed	l	anterior nasal: P	alive	1.06	1.94	**1902**
24	m	70	mixed	r	P	alive	1.99	2.06	**1022**
25	m	47	epithelioid	l	C	dead (liver)	1.53	2.08	**832**
26	m	42	epithelioid	r	P	alive	2.05	2.48	**947**
27	m	72	epithelioid	l	P	alive	1.97	2.48	**932**
28	f	68	epithelioid	l	juxtapapillary	alive	1.87	221	**965**
29	m	72	epithelioid	l	P	dead (liver)	1.88	2.27	**29**
30	m	64	spindle	l	anterior retinal: P	alive	1.23	2.22	**2021**
31	m	42	epithelioid	r	P	dead (orbita)	2.01	2.82	**303**
32	f	68	epithelioid	r	P	dead (liver, lung)	1.66	2.55	**439**
33	m	51	spindle-B	l	C	alive	0.94	2.14	**1609**
34	f	50	spindle-B	r	juxtapapillary	alive	2.22	2.50	**1097**
35	m	56	ND	l	anterior temporal: P	alive	1.33	2.37	**648**
36	f	55	epithelioid	l	anterior	alive	2.07	2.04	**623**
37	f	83	spindle-A	r	nasal: P	dead (liver)	1.40	1.80	**261**

38	f	63	spindle-A	r	C	alive	1.31	2.10	**490**
39	m	70	spindle-B	r	temporal: P	alive	1.17	2.33	**950**
40	f	61	spindle	l	P	alive	1.88	2.04	**740**
41	m	70	epithelioid	l	P	alive	1.41	1.81	**524**
42	f	70	epithelioid	r	P	alive	1.35	2.26	**582**
43	f	71	mix	r	anterior	alive	1.76	2.28	**559**
44	f	52	mix	r	temporal: P	alive	1.93	2.52	**560**
45	f	ND	spindle	l	C	alive	1.93	2.99	**592**
46	f	54	spindle	r	anterior temporal: P	alive	1.84	1.91	**613**

CI3: chromosome index 3, CI4: chromosome index 4, f: female, m: male, ND: no data, l: left, r: right, C: corpus ciliare, P: posterior pole

In the survival column, the cause of death (metastasis) is mentioned in brackets.

5.4 RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated using Nucleospin RNA and Protein purification kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. RNA from each sample (2000 ng) was reverse transcribed to cDNA using Tetra cDNA synthesis kit (Bioline, UK) in a final volume of 20 µl. In order to evaluate the expression of type I LH-RH receptors and LH-RH ligand, primer sets were designed. Gene-specific primers for LH-RH-I receptor: sense 5'-GACCTTGTCTGGAAAGATCC-3' (EXON 1 1844-1863), antisense 5'-CAGGCTGATCACCACCATCA-3' (EXON 1 1844-1863), for LH-RH ligand: sense 5'-GGCCTTATTCTACTGACTTGG-3', antisense 5'-TCTTCTGCCCAGTTTCCTCT-3'. HPRT1 was used as an internal reference gene. (sense: 5'-GTATTCATTATAGTCAAGGGCATATCC-3', antisense: 5'-AGATGGTCAAGGTCGCAAG -3'). mRNA levels of LH-RH-R-I, LH-RH and HPRT1 have been assessed using iQ™ SYBR® Green Supermix (BIO-RAD, USA). Reactions were conducted according to the manufacturer's protocol using MyiQ2 two color real time PCR detection system (BIO-RAD, USA). All real-time amplifications were measured in triplicates. Results were evaluated with BIO-RAD iQ5 (BIO-RAD, USA) software and changes in mRNA levels were calculated using the 2^{40-Ct} method.

5.5 Immunocytochemistry

5.5.1 Immunoperoxidase staining

To detect LH-RH-I receptors, OCM-1 and OCM-3 cells were fixed in ice-cold methanol (10 minutes). Endogenous peroxidase activity was blocked in 3 % hydrogen peroxide (10 min). Samples were permeabilized with 0.1 % Triton X-100 and blocked with 1 % BSA - 1 % FBS solution in 0.1% Triton X-100 (room temperature, 1 hour). Samples were incubated with primary anti-LH-RH-R antibody (sc-13944 rabbit polyclonal Santa Cruz, USA, 1:50) (overnight, 4°C) and EnVision Flex, HRP (Agilent Technologies, USA) (room temperature, 1 hour). Signals were detected using ready-to-use DAB substrate kit (Agilent Technologies, USA). Samples were rinsed with tap water, dehydrated through a graded series of alcohol, and mounted with ProLong® Diamond Antifade Mountant (Molecular probes, USA).

5.5.2 Immunofluorescent labeling

To investigate LH-RH-I receptors, OCM-1 and OCM-3 cells were fixed in 4 % paraformaldehyde at room temperature for 10 min, permeabilized with 0.1 % Triton X-100 at room temperature for 1 hour and blocked with 5 % BSA in 0.1 % Triton X-100 solution at room temperature for 1 hour. Samples were incubated with primary anti-LH-RH-R antibody (sc-13944 rabbit polyclonal Santa Cruz, USA, 1:50) (overnight, 4°C) and anti-rabbit FITC secondary antibody (ThermoFisher Scientific, USA, 1:1000). Samples were rinsed and mounted with ProLong® Diamond Antifade Mountant with DAPI (Molecular probes, USA). Staining was evaluated using the Olympus FV-1000 confocal microscope (Olympus Corporation, Japan).

5.6 Western blot

Total protein was isolated using Nucleospin RNA and Protein purification kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Total protein amount of the supernatant was determined by Nanodrop ND-1000 UV-Vis Spectrophotometer (USA). Equal amount of proteins (20 μg) were separated in 10 % SDS-polyacrylamide gels and then transferred to PVDF membrane using standard procedures (94). Upon blocking with 5 % BSA, membranes were incubated with primary antibodies (overnight, 4°C): anti-LH-RH-R (sc-13944 rabbit polyclonal Santa Cruz, USA 1:200) and anti-GAPDH (D16H11 rabbit monoclonal Cell Signaling 1:1000). Proteins were detected with anti-rabbit horseradish peroxidase conjugated antibody (mouse sc-2357 Santa Cruz, USA) and Luminata Forte Western HRP substrate (Merck Millipore, Germany). The protein bands were quantified using Image Lab software (Bio-Rad Laboratories, USA).

5.7 Preparation of membranes and radioligand binding studies

Preparation of membranes for receptor studies was performed as described previously (8,78,79). Receptor binding was characterized using sensitive *in vitro* ligand competition assay based on the binding of [^{125}I][D-Trp^{6}]-LH-RH as radioligand to membrane homogenates (8,78,79). The binding characteristics of receptors for LH-RH-I were determined in membrane fraction of OCM-1 and OCM-3 human UM cell lines (1.8-2.4 x 10^{8}

cells each) and in OCM-1 and OCM-3 tumors grew in nude mice. Radioiodinated derivatives of [D-Trp^6]-LH-RH were prepared by the chloramine-T method and purified by reverse-phase HPLC as described previously (78,79). This radioligand was well-characterized and showed high affinity binding to LH-RH-I receptors expressed in human and rat pituitaries and human breast, prostate, and other cancers (4,45,78–80). Briefly, membrane homogenates containing 50-160 μg protein were incubated in duplicate or triplicate with 60-80.000 cpm [^{125}I][D-Trp^6]-LH-RH and increasing concentrations (10^{-12} - 10^{-6} M) of nonradioactive peptides as competitors in a total volume of 150 μl binding buffer. At the end of incubation time, 125 μl aliquots of suspension were transferred onto the top of 1 ml of ice-cold binding buffer containing 1.5 % BSA in siliconized polypropylene microcentrifuge tubes (Sigma-Aldrich, USA). The tubes were centrifuged at 12.000x g, 4 ºC for 3 minutes. Supernatants were aspirated and the bottom of the tube containing the pellet was cut off and counted in a gamma counter. Protein concentration was determined by Bradford method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, USA). The LIGAND-PC computerized curve-fitting program of Munson and Rodbard was used to determine the type of receptor binding, dissociation constant (K_d) and maximal binding capacity of the receptors (B_{max}) (8,78,79).
This experiment was carried out with the help of my supervisor, professor Gabor Halmos.

5.8 Fluorescence in situ hybridization

5.8.1 Touch preparations

The tumor tissues were transferred from -80 to -20°C. The tissue samples were used for touch preparations, which were obtained by pressing frozen tissue samples several times on the surface of a silanized slide. The slides were fixed in methanol-acetic acid (3:1), air dried, washed with 70 % acetic acid solution and distilled water, dehydrated with 70 %, 80 % and 90 % ethanol and air dried. The slides were stored at -20°C until further use.

5.8.2 DNA FISH probes

Numerical aberrations of chromosome 3 and 4 were studied by FISH with CEP (Chromosome Enumeration DNA FISH Probes, Vysis, Germany). The probes consist of chromosome 3 or 4-

specific tandem-repeat DNA sequences. The CEP probes are directly labeled with SpectrumOrange (chromosome 3) and SpectrumGreen (chromosome 4) fluorophores. The centromeric probes contain 7 µl CEP Hibridization Buffer, 1 µl probe and 1 µl distilled water.

5.8.3 FISH hybridization

FISH was done according to a general protocol with some modifications (95). The slides containing the touch preparations were fixed in methanol-acetic acid (3:1) at -20°C, then incubated in 15 µl 10 % pepsin in 100 µl 1 M HCl. The slides were washed with 1x PBS buffer and then dehydrated in 70 %, 85 %, 100 % alcohol series and air dried. DNA FISH probe was added, coverslips were applied and sealed to the slide with rubber cement. The slides were denatured at 75°C for 5 minutes and hybridized at 42°C overnight. After hybridization, the slides were washed with 50 % formamide / 2xSSC solution at 42°C for 7 minutes then with 2xSSC solution at 42°C for 7 minutes. Slides were then counterstained with ProLong® Diamond Antifade Mountant with DAPI (Molecular probes, USA)

5.8.4 Fluorescence microscopy

Slides were evaluated using a fluorescence microscope (Axio Imager Z2, Zeiss, Germany). Image capture was performed by a monochrome charge-coupled device camera attached to the fluorescence microscope and ISIS software (Metasystems, Germany).

5.8.5 FISH analysis

Numerical aberrations of chromosome 3 and 4 were assessed by analysing chromosome copy number on the basis of 100 relevant tumor cell nuclei. CI values for chromosome 3 and 4 were determined for the ratio of the whole FISH signal in the sample and the number of nuclei. Chromosome loss was stated below 1.75, polysomy was stated above 2.25 chromosome copy number per nucleus. „Dominant" cell population value was determined. A cell population with a certain chromosome copy number was considered as „dominant" cell population where the cut-off limit was 15 % (12).

5.9 Statistical analysis

Correlation analysis was carried out between the expression of mRNA for LH-RH-I receptor and LH-RH ligand with the use of GraphPad Prism 7 (USA). The two data sets were evaluated using KS test, and then Pearson correlation analysis was performed.

Indices for chromosome 3 and 4 were analysed from the UM samples. The two data sets were evaluated using D'Agostino & Pearson omnibus normality test, and then Spearman correlation analysis was performed. Chromosome results, receptor findings and clinicopathological data were also analysed. Statistical analysis was carried out with the use of GraphPad Prism 7 (USA).

Survival data was plotted against the postoperative days (elapsed until death or the end of the follow-up period), according to the Kaplan-Meier method. Differences among the groups were investigated by means of Mantel-Cox log-rank test and Gehan-Breslow-Wilcoxon test. Statistical analysis was carried out with GraphPad Prism 7 software (USA).

6 Results

6.1 Expression of type I LH-RH receptor in human UM *in vitro*.

mRNA expression of LH-RH-I receptors has been analyzed in OCM-1 and OCM-3 cell lines by RT-qPCR. LH-RH-I receptors were detected in both these human UM cell lines, with slightly higher expression of LH-RH-I receptor observed in OCM-3 cells (Figure 10 A). Western blot analysis confirmed the presence of LH-RH-I receptors in OCM-1 and OCM-3 cells and revealed a signal corresponding to a protein of approximately 68 kDa, which is the exact molecular mass of LH-RH-I receptor (96) (Figure 11). In accordance with the receptor mRNA data, a slightly higher protein expression of LH-RH-I receptors was observed in OCM-3 cells by Western blot and immunocytochemical analysis (Figure 12).

6.2 Expression of type I LH-RH receptors *in vivo* in tumor xenograft models

The mRNA expression of LH-RH-I receptors in OCM-1 and OCM-3 tumors grown in nude mice was analyzed by RT-qPCR. mRNA expression of LH-RH-I receptors could be detected in all tumor xenografts (Figure 10 A). In accordance with our *in vitro* results, the level of LH-RH-I receptor transcript in our OCM-3 model was considerably higher than in the OCM-1 tumor samples. Western blot analysis also confirmed the presence of LH-RH-I receptor protein in OCM-1 and OCM-3 tumor tissues. Similarly to our *in vitro* findings, Western blot analysis confirmed higher protein expression levels of LH-RH-I receptor in OCM-3 tumor xenografts (Figure 11).

6.3 Expression of mRNA for LH-RH in human UM cell lines and tumor xenografts

In addition to LH-RH receptor studies, the expression of LH-RH ligand in OCM-1 and OCM-3 cellular models was also investigated by RT-qPCR. The presence of mRNA of LH-RH ligand was detected in both cell lines and tumors grown in nude mice (Figure 10 B). Although the expression of LH-RH-R was considerably higher in OCM-3 cells, the mRNA expression of LH-RH ligand was slightly higher in OCM-1 cells (Figure 10 B).

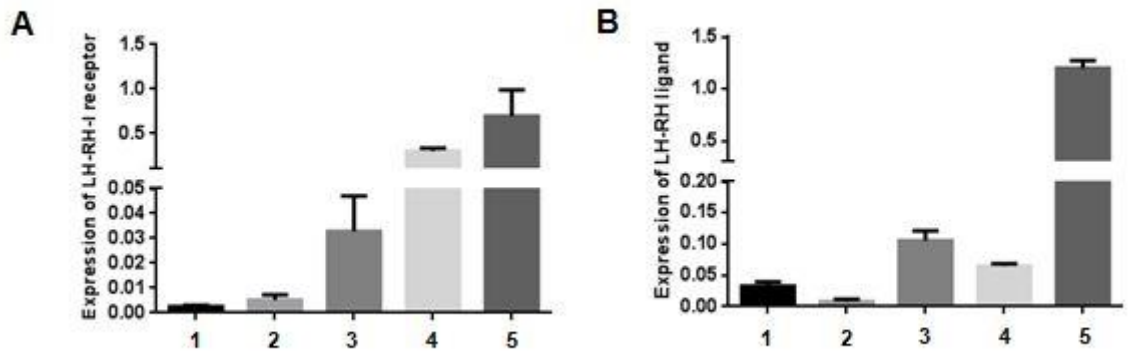

Figure 10. RT-qPCR analysis of the of LH-RH-I receptor (A) and LH-RH ligand (B) in human UM cell lines: (1) OCM-1, (2) OCM-3, (3) xenografted OCM-1 (4) xenografted OCM-3 (5) positive control, human pituitary. Y-axis represents fold change in gene expression, normalized to HPRT1 gene. Data represent mean values ± SE (n=3).

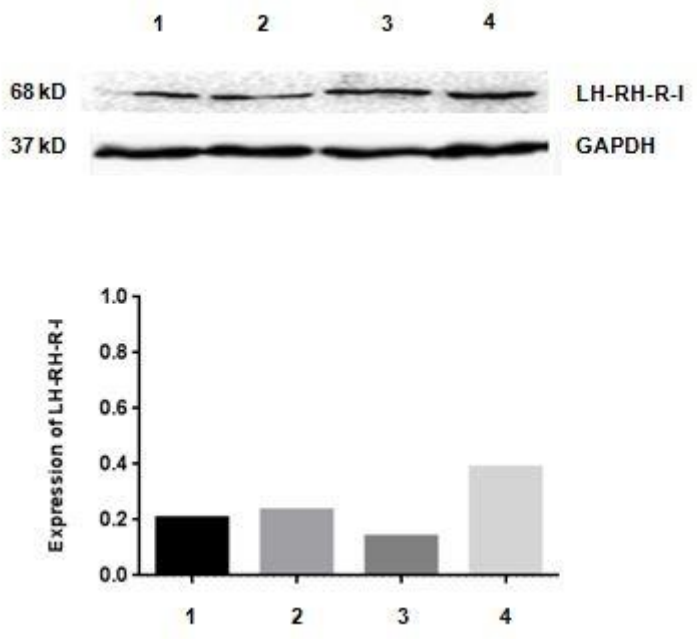

Figure 11. Western blot analysis of LH-RH-I receptor in human UM cell lines: (1) OCM-1, (2) OCM-3, (3) xenografted OCM-1 (4) xenografted OCM-3. Signal density was quantified by densitometric scanning and normalized to that of GAPDH. Each value represents the mean of two technical replicates.

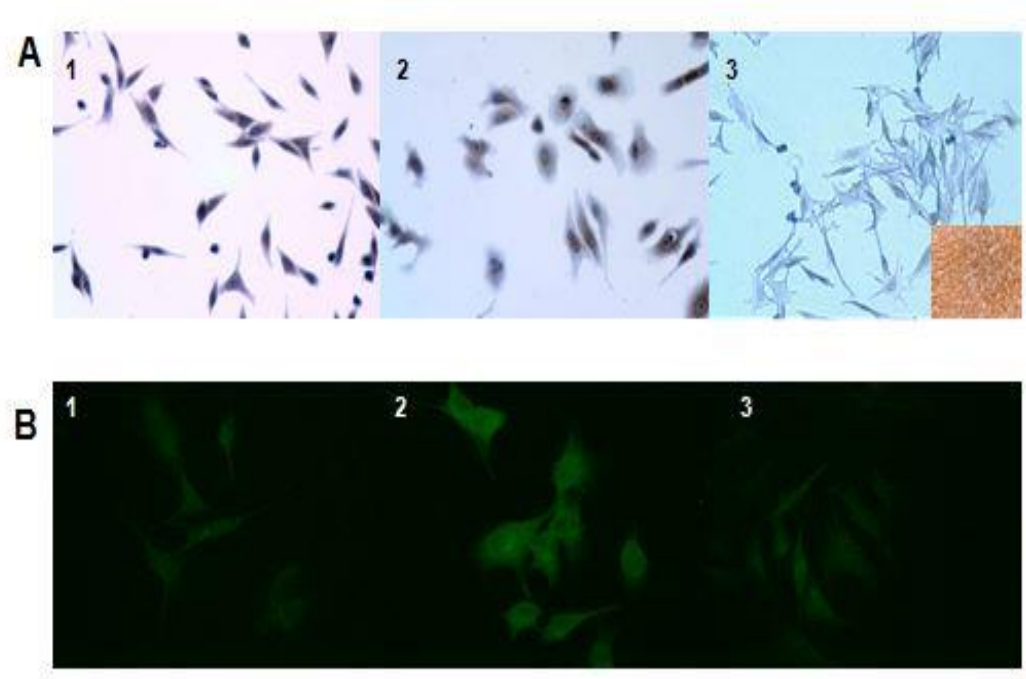

Figure 12. Immunocytochemical analysis of LH-RH-I receptor in OCM-1 and OCM-3 cells. (A) Immunoperoxidase staining: (1) OCM-1 (2) OCM-3 (3) No antibody control. Inset: positive control, human pituitary, DAB (brown) (magnification: 20x). (B) Immunofluorescent labeling: (1) OCM-1 (2) OCM-3 (3) No antibody control (magnification: 60x)

6.4 Radioligand Binding Studies

The presence of specific LH-RH binding sites and binding characteristics of [^{125}I][D-Trp^{6}]-LH-RH to membrane receptors in OCM-1 and OCM-3 human UM models were determined using ligand competition assays. Analysis of the typical displacement of radiolabeled [^{125}I][D-Trp^{6}]-LH-RH by the same unlabeled peptide revealed that the one-site model provided the best fit, indicating the presence of one class of high-affinity LH-RH-I receptors in crude membranes derived from human UM cells. In cell membranes of OCM-1 and OCM-3 cells, ligand competition studies revealed a single class of high affinity binding sites for LH-RH-I with a mean dissociation constants (K_d) of 4.11±0.3 nM and 4.26±0.6 nM, respectively (Table II). The concentration of LH-RH-I receptors was 233.6±21.7 fmol/mg membrane protein in OCM-1 cells while OCM-3 cells showed remarkably higher receptor level (1029.1±68.5 fmol/mg membrane protein) (Table II). Receptors for LH-RH-I have also been found in the membranes of OCM-1 and OCM-3 tumor tissue samples. Radiolabeled [^{125}I][D-Trp^{6}]-LH-RH

was found to be bound to a single class of specific, high affinity binding sites in both human UM models investigated. Mean K_d values were 5.85±0.7 nM in OCM-1 tumors and 6.18±0.8 nM for OCM-3 tumors (Table III). Mean B_{max} values were as the followings; 267.3±38.5 fmol/mg membrane protein in OCM-1 tumors and about 2.7 times higher (713.0±29.4 fmol/mg membrane protein) in OCM-3 xenografts (Table III). Biochemical parameters, which are essential to establish the identity of specific binding sites, were also determined. The binding of [^{125}I][D-Trp^6]-LH-RH was found to be reversible, time- and temperature-dependent, and linear with protein concentration in human UM samples. The specificity of LH-RH binding was demonstrated by competitive binding experiments using several peptides structurally related or unrelated to LH-RH. The binding of radiolabeled [^{125}I][D-Trp^6]-LH-RH was completely displaced by increasing concentrations (10^{-12} - 10^{-6} M) of LH-RH agonist buserelin and LH-RH antagonist cetrorelix (data not shown).

Table II. Binding characteristics of LH-RH-I receptors in OCM-1 and OCM-3 human UM cells

Cell line	K_D (nM)	B_{max} (fmol/mg protein)
OCM-1	4.11 ± 0.3	233.6 ± 21.7
OCM-3	4.26 ± 0.6	1029.1 ± 68.5

K_D: dissociation constant, B_{max} : maximal binding capacity

Binding characteristics were obtained from ligand competition assays, based on the specific binding of radiolabeled [^{125}I][D-Trp^6]-LH-RH to membrane homogenates. All values represent mean ± SE (n=3).

Table III. Binding characteristics of LH-RH-I receptors in OCM-1 and OCM-3 human UM xenografted into nude mice

Cell line	K_D (nM)	B_{max} (fmol/mg protein)
OCM-1	5.85 ± 0.7	267.3 ± 38.5
OCM-3	6.18 ± 0.8	713.0 ± 29.4

K_D: dissociation constant, B_{max} : maximal binding capacity

Binding characteristics were obtained from ligand competition assays. Based on the binding of radiolabeled [^{125}I][D-Trp^6]-LH-RH to membrane homogenates. All values represent mean ± SE (n=3).

6.5 Correlation between type I LH-RH receptor and LH-RH ligand expressions

According to our statistical analysis, there is a significant correlation between the expression of mRNA for LH-RH-I receptor and LH-RH ligand in OCM-1 cell line and in OCM-1 tumor xenografts (Pearson r=0.8380; p=0.0373, CI=0.95 %). Moreover, significant correlation was also observed between the expressions of LH-RH-R-I and LH-RH ligand in OCM-3 cells and OCM-3 tumors grown in nude mice (Pearson r=0.9878; p=0.0002, CI=0.95 %) (Figure 13).

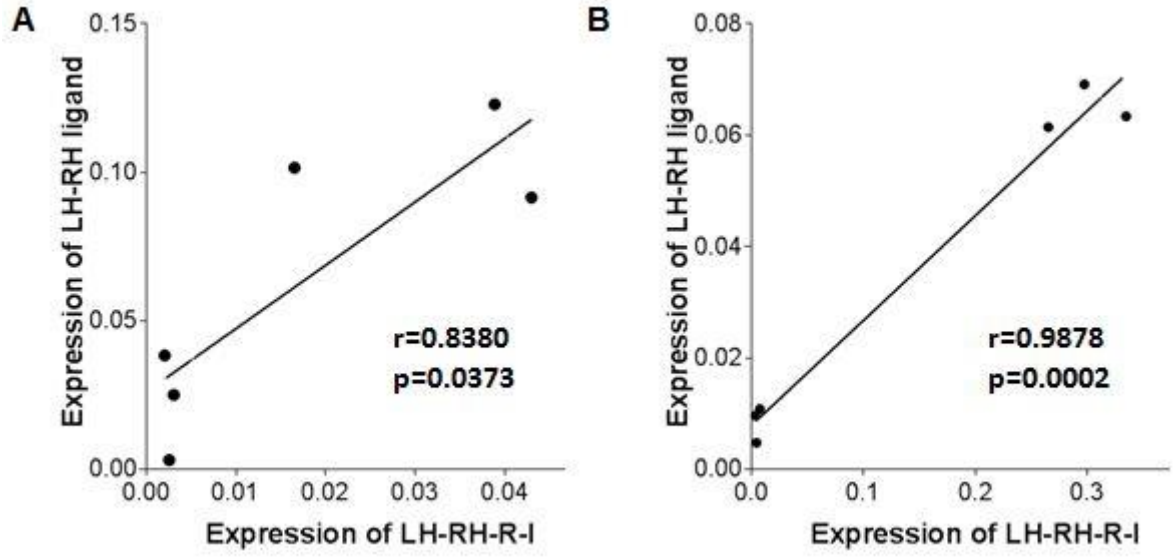

Figure 13. Correlation between the mRNA expression of LH-RH-I receptor and LH-RH ligand in OCM-1 **(A)** and in OCM-3 **(B)** cells.

6.6 Distribution of chromosome 3

Based on CI values, monosomy of chromosome 3 could be found in 16 (35 %) samples. In 6 specimens (13 %), more than 2 copies of chromosome 3 have been found. Normal biparental disomy was observed in 24 samples (52 %). In 26 samples one signal per cell per „dominant" cell population could be detected, whereas in 9 cases, clones containing 3 or more chromosomes per nucleus have been found. In two specimens, either loss of chromosome 3 or polysomy have been observed. Normal distribution of chromosome 3 was detected in 13 cases. In addition, normal tissue samples contained negligible abnormal cell population (<15 %) (Table IV). Representative distribution of chromosome 3 is shown in Figure 14.

Table IV. Distribution of chromosome 3 in human UM specimens.

Sample ID	Chromosome 3				
	„Dominant" cell population 1		„Dominant" cell population 2		Chromosome index (CI)
	Signal/cell	%	Signal/cell	%	
33	1	85 %			0.94
23	1	94 %			1.06
39	1	78 %			1.17
30	1	77 %			1.23
38	1	69 %			1.31
35	1	71 %			1.33
42	1	66 %			1.35
13	1	65 %			1.37
19	1	64 %			1.37
37	1	62 %			1.40
41	1	62 %			1.41
1	1	62 %			1.43
10	1	60 %			1.48
25	1	52 %			1.53
32	1	39 %			1.66
14	1	49 %			1.71
43	1	25 %			1.76
20	1	34 %			1.79
46	1	23 %			1.84
28	1	21 %			1.87
29	1	19 %			1.88
40	1	17 %			1.88
44	normal				1.93
45	normal				1.93
27	1	21 %			1.97
24	normal				1.99

2	1	35 %			2.00
31	normal				2.01
7	normal				2.04
9	normal				2.04
26	normal				2.05
11	normal				2.07
17	normal				2.07
36	3	17 %			2.07
12	1	24 %	3	18 %	2.10
6	normal				2.17
5	normal				2.18
3	normal				2.19
8	normal				2.21
34	3	21 %			2.22
21	3	19 %			2.26
18	1	42 %	≥4	27 %	2.28
4			3	18 %	2.41
22	3	20 %			2.43
16	3	18 %	≥4	18 %	2.53
15	3	29 %	≥4	22 %	2.68

A cell population with a certain chromosome copy number was considered as „dominant” cell population where the cut-off limit was 15 %. The samples are listed according to their CI value.

6.7 Distribution of chromosome 4

Based on CI values, chromosome 4 could be detected in normal biparental disomy in 14 samples (30 %), while 32 cases (70 %) showed more than 2 signals per nucleus. In 8 samples one signal per cell per „dominant" cell population has been observed, whereas in 41 cases, clones containing 3 or more chromosomes per nucleus have been found. In 6 specimens either loss of chromosome 4 or polysomy has been observed. Normal distribution of chromosome 4 was detected only in 3 cases (Table V). Representative distribution of chromosome 4 is shown in Figure 14.

Table V. Distribution of chromosome 4 in human UM specimens.

Sample ID	Chromosome 4				
	„Dominant" cell population 1		„Dominant" cell population 2		Chromosome index (CI)
	Signal/cell	%	Signal/cell	%	
17	1	22 %			1.75
37	1	44 %	3	24 %	1.80
41	1	39 %	3	20 %	1.81
16	1	15 %			1.91
46	1	26 %	3	17 %	1.91
23	normal				1.94
36	1	23 %	3	24 %	2.04
40	1	30 %	3	32 %	2.04
24	normal				2.06
25	normal				2.08
38	1	20 %	3	28 %	2.10
33	3	21 %			2.14
28	1	16 %	3	21 %	2.21
30	3	26 %			2.22
42	3	36 %			2.26
29	3	26 %			2.27
43	3	38 %			2.28
19	3	15 %			2.31
39	3	44 %			2.33
22	3	20 %			2.36
35	3	32 %			2.37
20	3	18 %			2.39
26			≥4	18 %	2.48
27	3	25 %			2.48
34	3	37 %			2.50
44	3	34 %			2.52
12	3	20 %			2.53

32	3	42 %			2.55
2			3	16 %	2.65
1			≥4	30 %	2.72
10	3	25 %	≥4	22 %	2.79
8	3	45 %			2.81
31	3	48 %			2.82
14	3	19 %	≥4	33 %	2.94
45	3	58 %	≥4	21 %	2.99
4	3	26 %	≥4	26 %	3.00
15	3	19 %	≥4	39 %	3.03
21	3	79 %			3.04
6	3	28 %	≥4	42 %	3.34
3	3	27 %	≥4	43 %	3.39
11	3	28 %	≥4	47 %	3.43
18	3	15 %	≥4	24 %	3.43
5	3	19 %	≥4	72 %	3.94
9			≥4	81 %	3.94
7	3	22 %	≥4	67 %	4.01
13			≥4	91 %	5.39

A cell population with a certain chromosome copy number was considered as „dominant” cell population where the cut-off limit was 15%. The samples are listed according to CI value.

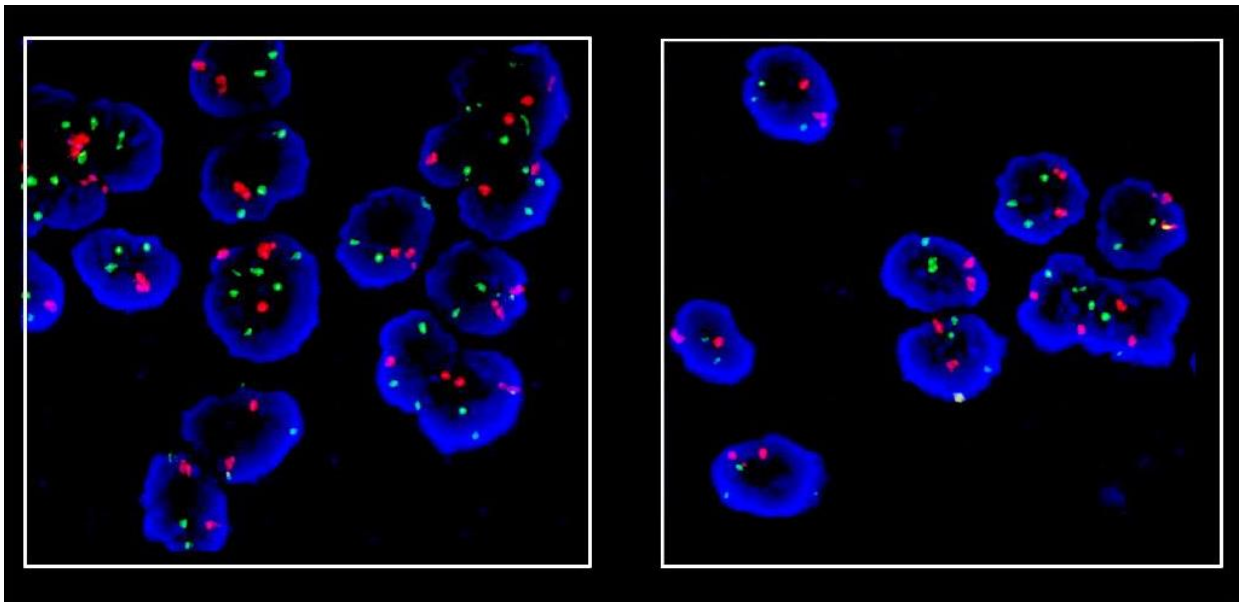

Figure 14. Representative picture of FISH analysis in human UM. Nuclei were stained with blue fluorescent DAPI. Specific signs of chromosome 3 are represented as red signals. Green signals show specific signals of chromosome 4.

6.8 Statistical results

According to the statistical analysis, there is a statistically significant ($p<0.05$) correlation between the copy number of chromosome 3 and 4 (Spearman r=0.42; 0.139-0.639; CI=0.95%) (Figure 15).

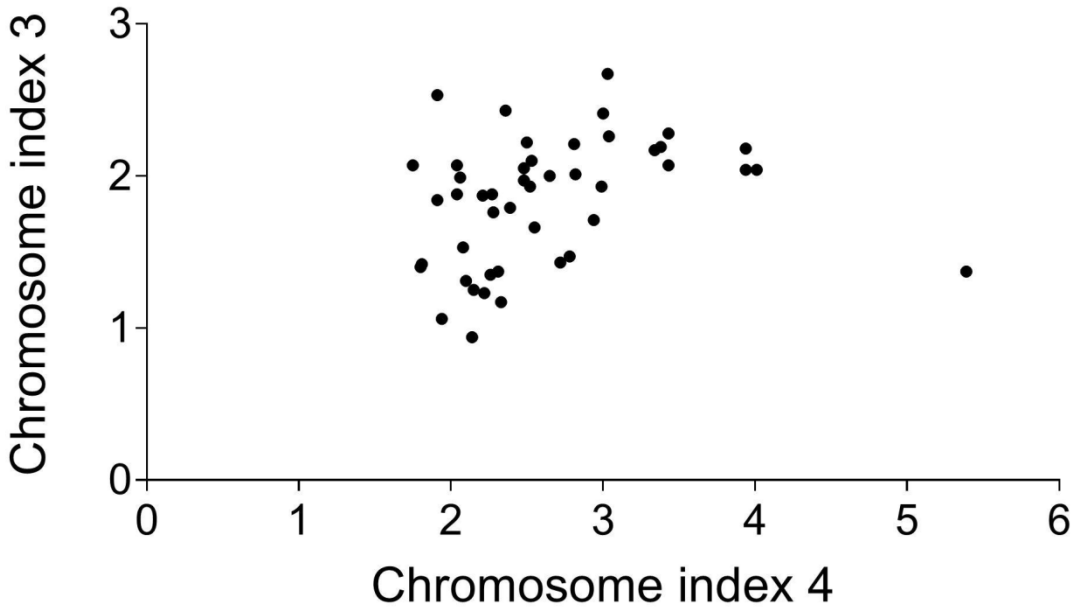

Figure 15. Correlation between the copy number of chromosome 3 and 4 in 46 human UM specimens. There is a significant (p=0.0036) correlation between the copy number of chromosome 3 and 4 (Spearman r=0.42; 0.139-0.639; CI=0.95 %)

CI values of chromosomes 3 and 4 were determined for the samples and were considered to be normal (N: 1.75-2.25) or pathological (P: <1.75 or >2.25). Comparing the survival rate of the four groups (NN, NP, PN, PP), obvious difference has been revealed, however, statistically significant differences could not be shown (p=0.38 for the Mantel-Cox test and p=0.43 for the Gehan-Breslow-Wilcoxon test). Even the two major groups (N, P) have not been found to be significantly different (p=0.12 by both the Mantel-Cox and Gehan-Breslow-Wilcoxon tests), in spite of the considerable difference between their survival curves (Figure 16). The correlation between chromosome 3 and 4 aberrations and LH-RH-R was also investigated in 17 UM samples where receptor data were available (8). No significant correlation was found between the chromosome copy number and the expression and binding characteristics of LH-RH-R. Furthermore, based on our findings and the clinicopathological data, there is no correlation between the clinical outcome and chromosome 3 and 4 status (Table VI).

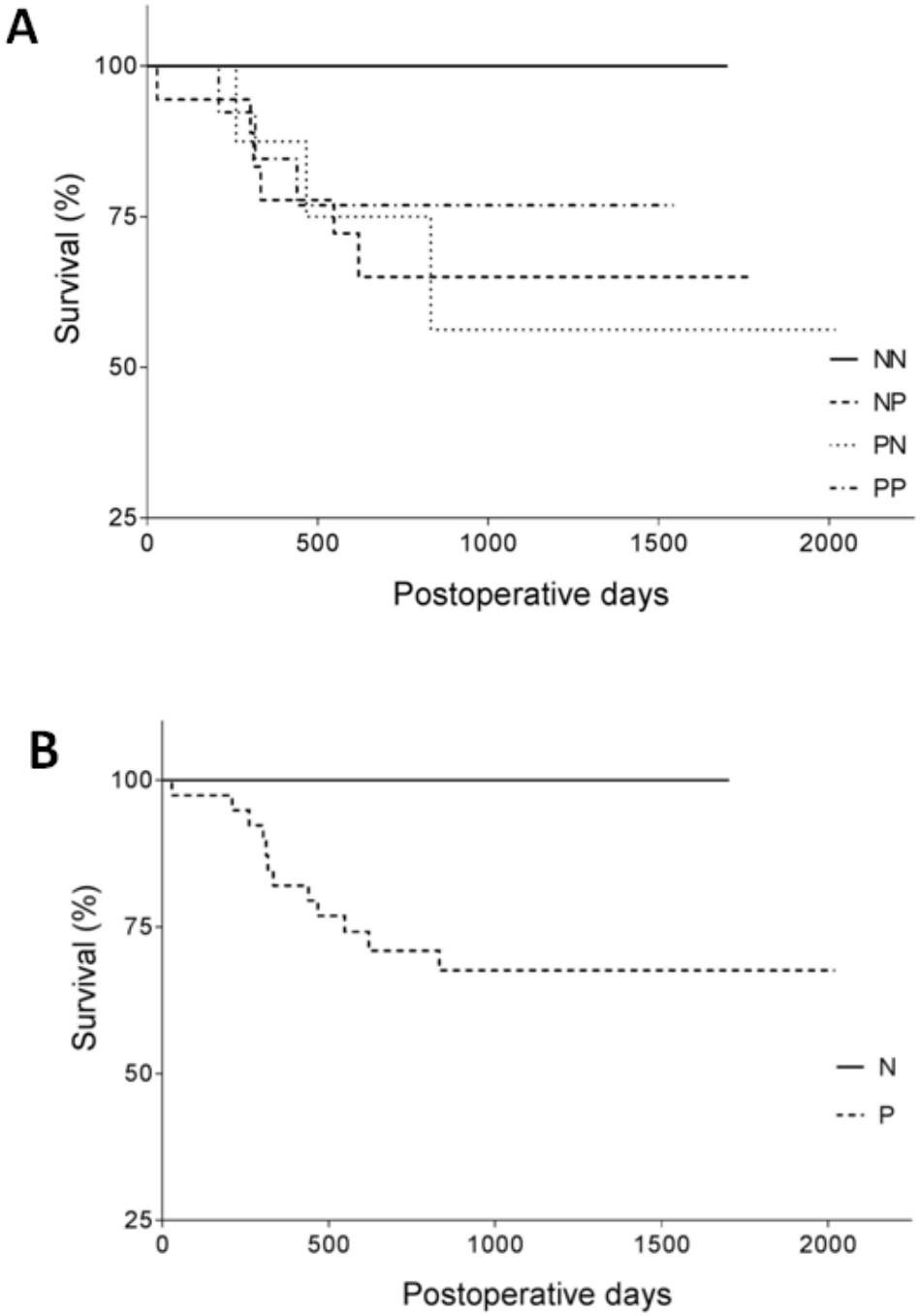

Figure 16. Kaplan-Meier survival curves of patients with UM A) NN (normal CI3 and CI4), NP (normal CI3 pathological CI4), PN (pathological CI3 and normal CI4) and PP (pathological CI3 and CI4) status. (p=0.38 for the Mantel-Cox test and p=0.43 for the Gehan-Breslow-Wilcoxon test) B) N (including NN) and P (containing NP, PN and PP) status. (p=0.12 for both the Mantel-Cox and Gehan-Breslow-Wilcoxon tests)

Table VI. Clinicopathological characteristics, LH-RH-I receptor and ligand expression results and survival data of 17 UM patients

ID	Sex	Age	Type	Eye	Localization	Survival	LH-RH-R-I expression	LH-RH expression
3	f	44	spindle-B	l	inferior temporal: P	alive	-	+
4	f	50	spindle	r	temporal: P	alive	+	-
5	m	76	spindle	r	P	dead (liver)	+	+
6	f	30	spindle-A	l	P	alive	+	+
7	m	66	epithelioid	l	temporal: P	alive	+	+
8	m	61	spindle-B	l	temporal: P	alive	-	+
9	m	53	ND	l	superior temporal: P	alive	-	-
10	m	53	epithelioid	r	P	alive	-	+
11	f	79	epithelioid	r	P	dead	+	-
12	m	67	epithelioid	l	P	alive	+	+
13	f	72	epithelioid	l	temporal: P	dead (liver)	-	+
14	m	35	spindle	l	superior nasal: P	alive	-	-
15	m	55	spindle-B	l	P	alive	-	+
16	m	65	spindle-B	r	anterior temporal: P	dead	+	-
17	f	68	spindle	l	P	alive	-	+
33	m	51	spindle-B	l	C	alive	-	+
34	f	50	spindle-B	r	juxtapapillary	alive	+	-

f: female, m: male, ND: no data, l: left, r: right, C: corpus ciliare, P: posterior pole
In the survival column, the cause of death (metastasis) is mentioned in brackets.

7 Discussion

UM is the most common primary intraocular tumor of the eye. Major risk factors for the development of human UM are – among others – dysplastic naevus syndrome, Caucasian ethnicity, ocular melanocytosis and light eye color (97). Mainly in the liver (~95 %), approx 40-50 % of the patients with primary UM develop metastases (98). Patients have a median survival of about 6-9 months once a metastasis develops (99,100). So far, there is no effective adjuvant systemic therapy to prolong patient survival (101). Therefore, the investigating of new therapeutic approaches and a better understanding of the molecular background of UM are urgently needed.

Growing body of evidence shows that LH-RH receptors can serve as a potential therapeutic target (4,44–46,69,78–80,102). During the past decades great number of studies and deep scientific work focused on the investigation of the expression of LH-RH receptors. This field of research generated hundreds of peer reviewed publications demonstrating the presence of this type of receptor in various animal and human cancers. In these investigations not only receptors for LH-RH but specific, high affinity receptors for other hypothalamic hormones were also studied by several groups. The effects of LH-RH and its analogs are mediated for LH-RH by high-affinity GPC receptors located in the membranes of the pituitary gonadotrophs and different human tumor cells (4,45,78,79,103). In vertebrates, three subtypes of LH-RH receptors have been classified (75,77,104–107). Mainly, type I receptors are located in the pituitary and mediate the regulation of gonadotropin secretion (75,77,104–106). In humans, type II LH-RH receptors have become nonfunctional (75,104). Their function - being the target for LH-RH-II - has been taken up by type I receptors (75,104). However, LH-RH-II activates type I receptors differently than LH-RH-I (106). Interestingly, it has been investigated that type II LH-RH receptors might play a role in cancer cell growth (105). Type III LH-RH receptors seem to be related to type II receptors, they might have common genetic roots (75). In the past decade several studies were published by various teams to show these new findings about the potential role of type I, type II and type III LH-RH receptors. However, more research is nesessary to see the importance and pathophysiological function of these receptor subtypes. LH-RH-I is mainly localized in the hypothalamus, however, it has also been observed in the ovary, endometrium, breasts, placenta, testis, kidneys, prostate thymus, and in lower levels in several other organs (4,45,46,69,78). The presence of specific, high affinity LH-RH-R-I in various human cancers and tumor cell lines originating from various organs other than those of the human reproductive system has been shown in several

studies (4,45,69,79,80,103,108). In our previous study we have demonstrated that 46 % of human UMs express receptors for LH-RH-I (8). Acting directly on the target cancer cells, both agonists and antagonists of LH-RH might serve as potential therapeutic agents (4,45,107–109). Hundreds of agonistic and antagonistic analogs of LH-RH have been synthesized for further therapeutic applications. After continuous exposure, LH-RH agonists inhibit the gonadotropin secretion (109). In contrast, receptor antagonists of LH-RH can produce a competitive blockade of specific LH-RH-R leading to an immediate cessation of the secretion of gonadotropins and sex steroids, compared to the agonists, reducing the time of the onset of therapeutic effects (5). Agonistic analogs, such as goserelin, triptorelin, buserelin and leuprolelin are used in human oncology and gynecology very extensively (4,45,69,80,108). Potent antagonists of LH-RH, such as ganirelix, degarelix, abarelix and cetrorelix have also been deeply investigated and are now available for human therapy (4,5,45,69,80,108). Specific receptors for LH-RH on various human cancers might also serve as molecular targets for novel LH-RH analogs coupled to cytotoxic radicals (4,45,46,79,81,82). During the last two decades the highest number of studies were published about two cytotoxic LH-RH analogs AN-152 and AN-207 demonstrating their antiproliferative effect *in vitro* and *in vivo.* Based on these investigations these compounds seem to be very promising antitumor drug candidates. In the analog AN-152 (AEZS-108) DOX is covalently bound to the LH-RH agonist D-Lys^{6}-LH-RH, that binds to the receptors with high affinity located on the surface of human prostate, breast, ovarian, endometrial and other tumor cells (4,45,46,81,82). This analog has been substantially developed in a large number of experimental studies (4,45,46,69,79–82,108) and also tested clinically in endometrial, ovarian, bladder and prostate cancer. In endometrial cancer, it is in clinical phase III trials (84).

In UM research, experimental cell lines have been widely used in order to characterize and identify the disease *in vitro* (110). Such *in vitro* models are essential to investigate a certain desease including the expression of novel molecular targets or study the mechanism of action of new potential therapeutic compounds. In the present study, our aim was to examine the mRNA expression of LH-RH-I receptors and LH-RH ligand in OCM-1 and OCM-3 human UM cell lines. Those cultures were derived from primary UMs (111). The presence and binding characteristics of LH-RH-I receptor protein was also examined by Western blot, immunocytochemistry and ligand competition assays. Furthermore, we have investigated the expression of LH-RH-I receptors at mRNA as well as protein levels in OCM-1 and OCM-3 models trasplanted into nude mice.

The expression of LH-RH-I receptors provides support to the therapeutic use of LH-RH analogs coupled to cytotoxic drugs in human UM as well as many other hormone dependent tumors (4,45,46,79,81,82,102). Since human cancer is a major health problem around the world we need to find novel therapeutic approaches to fight this disease. New molecular targets such as hypothalamic peptide hormone receptors should be identified and investigated. The concept of targeted therapy is very promising. Targeted tumor therapy decreases adverse reactions and peripheral toxicity compared to systemic chemotherapeutic agents, and increases selective damage to tumor cells (4,44–46,81,82,102). For example, a cytotoxic LH-RH analog, AN-152 (AEZS-108) widely used in targeted therapy. AN-152 consists of DOX coupled covalently to LH-RH agonist [D-Lys^6]-LH-RH and binds with high affinity to LH-RH receptors on the membrane of different tumor cells (4,7,45,46,79,81,82,96,102,108). Moreover the therapy with cytotoxic LH-RH analogs does not induce any cardiotoxicity and does not inflict permanent damage to pituitary function (7,112,113). AN-152 has been tested in phase II and III clinical trials in endometrial and ovarian cancers and in phase I/II trials in castration resistant prostate cancer (7,82,84,85). Modern LH-RH analogs including agonistic analogs or antagonists such as Degarelix could also be used. More studies are needed to demonstrate the antitumor effect of these potential drug candidates.

The results of this study lend evidence for the present of LH-RH-I receptors in two human UM cell lines and show that OCM-3 cells express LH-RH-I receptors at higher level than OCM-1 cells. Our findings are very novel since this is the first demonstration of the presence of receptors for LH-RH in human experimental UM models. The same expression motive has been detected in our *in vivo* models. Moreover, a notable mRNA expression of the LH-RH ligand was observed in both cell lines and tumor tissues grown in nude mice. Significant correlation was observed between the LH-RH ligand and LH-RH-I receptor expression in OCM-1 and OCM-3 cell lines. The existence of LH-RH-I receptor protein was evaulated by Western blot in both cell lines cultured *in vitro* and tissue samples from nude mice. In addition, using ligand competition assay we determined the binding of [^{125}I][D-Trp^6]-LH-RH to membrane preparations of OCM-1 and OCM-3 human UM models. In both human UM models investigated, specific high affinity LH-RH-I receptors were found. The investigation of binding characteristics (affinity, concentration, specificity) of these membrane bound receptors together with other protein based receptor evaluations can provide very important information for further studies. The expression of LH-RH ligand and co-expression of LH-RH-I receptors might provide some evidence for an autocrine and/or paracrine regulatory

system in human UM. Our results further support the hypothesis, that locally produced LH-RH may participate in the regulation of tumor growth (4,45,103,108,114,115).

Generally, the genetic background of various tumors has been developed substantially. During the past decades great number of studies and valuable scientific work focused on the investigation of the genetic background of human cancer. E.g. aberrations of chromosome 4 have been demonstrated in small cell lung cancer, cervical cancer, chronic lymphocytic leukemia gliobastoma (116–119). Chromosome 4 hyperploidy is the most prominent aberration found in Barrett's metaplasia and 89 % of the patients display this alterations (120). Interestingely, the gene encoding LH-RH-R is harbored on chromosome 4q21.2. However, the numerical alterations of chromosome 4 have never been studied in UM. Based on our knowledge our work is the first one to investigate this field.

It has been reported that loss of one copy of chromosome 3 strongly correlates with metastatic risk and other chromosomal alterations firmly predict the risk of a metastatic disease (21,31). Monosomy 3 in choroidal melanoma is a significant predictor of metastasis-related death and has been correlated with a 70 % decrease in 5 year survival. Infrequently, alterations of other chromosomes such as losses of 1p, 6q, 9p, 10, 11q23–q25, and gain of chromosomes 6p, 7, 8q and 10 have been described (21,34). *GNAQ, DDEF1, NBS1, HDM2, BCL-2*, and *CCND1* have been proposed as potential genes in UM recently. However a significant role of most of these genes must be further developed in tumorigenesis and progression towards metastasis has to be confirmed (121,122).

In this study, our aim was to examine the copy number of chromosome 3 since it has been involved in the aggressive behavior of UM. More importantly, copy number of chromosome 4 has also been investigated in the same human UM specimens using FISH. The correlation between LH-RH-R expression, numerical alterations of chromosome 3 and 4 and clinicopathological findings has similarly been examined.

We show here for the first time, that chromosome 4 is present in an abnormal copy number in the majority of UMs. Based on the CI values 2 normal copies have been found only in 30 % of cases, while in 70 % of samples of chromosome 4 more than 2 signals per nucleus were detected. Loss of one copy of chromosome 3 has been evaluated in 35 % of samples while in 13 % of the cases polysomy has been detected. Our results are somewhat different from previous reports about the frequency of the monosomy of chromosome 3 (50 %) (9,10,123,124). This slight difference might be partially explained by the possibly diverse genetic background of the Hungarian population.

In case of chromosome 3, based on „dominant" cell population values, one signal per cell per „dominant" cell population has been detected in 26 samples whereas we could observe clones containing 3 or more chromosomes per nucleus in 9 cases. In two specimens either monosomy or polysomy has been detected.

In case of chromosome 4, in 41 cases clones containing 3 or more chromosomes/nucleus have been observed, whereas one signal per cell per „dominant" has been detected in 8 samples. In 6 specimens either loss of the chromosome or polysomy has been shown.

According to our statistical analysis, there is a moderate, statistically significant correlation between the copy numbers of chromosome 3 and 4, but no correlation was found with LH-RH-R expression and chromosome aberrations.

We also evaluated the survival rate of the UM patients according to their CI. Comparing the survival rate of the four groups (NN, NP, PN, PP) and the two major groups (N, P) moderate difference has been observed, although statistically significant differences could not be proven in spite of the remarkable difference between their survival curves. As mentioned above, the limited number of human UM specimens as well as the diverse genetic background of the Hungarian population might have contributed to the limitation of our study.

In summary, in the present thesis we observed the expression of LH-RH ligand and LH-RH-I receptor as a potential therapeutic target in two human UM cell lines and tumor xenografts grown in nude mice. Our findings support the development of new therapeutic agents based on cytotoxic analogs of LH-RH targeting LH-RH receptors in UM. In conclusion, our results provide new informations about the genetic background of UM and may lead to a more precise prognosis and novel therapeutic approaches of eye cancer.

8 Summary

Previously, we have demonstrated that approximately 50 % of UMs express LH-RH-R-I. The gene encoding LH-RH-R-I is located in chromosome 4, however the occurrent numerical aberrations of chromosome 4 have never been studied in UM.

In the present study, our aim was to examine the expression of mRNA for receptors of LH-RH-I and LH-RH ligand in OCM-1 and OCM-3 human UM cell lines. The presence and binding characteristics of LH-RH-I receptor protein was further evaluated by Western blot, immunocytochemistry and ligand competition assay. The mRNA and protein expression of LH-RH-I receptors have also been determined using cancer samples originating from nude mice xenografted with OCM-1 or OCM-3 cells. Moreover, we investigated the abnormalities of chromosome 3 and 4 and the possible correlation between them and with the expression of LH-RH-R. 46 UM specimens were obtained after enucleation. Numerical aberrations of chromosome 3 and 4 were studied by FISH.

mRNA expression of LH-RH-I receptor has been observed in OCM-1 and OCM-3 cell lines and was found higher in OCM-3 cells. LH-RH-I receptor mRNA was also investigated in both UM xenograft models with higher levels in OCM-3 xenografted mice. The existence of LH-RH-I receptor protein was found in both cell lines, and also in cancer tissue samples grown in nude mice. Both human UM models showed specific high affinity receptors for LH-RH-I using ligand competition assay. Expression of mRNA for LH-RH ligand has also been observed in both cell lines and tumor tissues. Chromosome 4 could be observed in normal 2 copies only in 14 samples (30 %), however, 32 cases (70 %) showed more than 2 copies. Loss of one copy of chromosome 3 could be observed in 16 samples (35 %). Normal biparental disomy could be detected in 24 samples (52 %), while in 6 specimens (13 %), more than 2 copies of chromosome 3 were found. Statistical analysis indicates significant correlation ($p<0.05$) between the copy number of chromosome 3 and 4. Moreover, moderate difference has been revealed in the survival rate of the UM patients with various pathological profiles. No correlation was found between LH-RH-R expression and chromosome aberrations. In summary, the expression of LH-RH-I receptors in OCM-1 and OCM-3 human UM cell lines suggests that these receptors could serve as potential molecular target for novel therapies. In conclusion, the results presented in the current dissertation could contribute to a more precise determination of the prognosis of human UM and to the development of new therapeutic approaches to this malignancy.

9 Összefoglalás

Korábbi vizsgálataink során az UM-ák jelentős részében (46%) detektáltuk az LH-RH-I receptorát. Az LH-RH-I receptor génje a 4-es kromoszómán helyezkedik el, azonban ezen kromoszóma szerepéről az UM kialakulásában és prognózisában nem áll rendelkezésre irodalmi adat. Kísérleteinkben célul tűztük ki az LH-RH ligand és a receptor mRNS szintű kimutatását OCM-1 és OCM-3 sejtvonalakban. A receptor fehérje jelenlétét immuncitokémiával és Western blottal, funkcióképességét és kötési karakterisztikáit ligand kötési assay-vel is megvizsgáltuk. Az LH-RH-I receptor mRNS és fehérje szintű expresszióját OCM-1 és OCM-3 egér xenograft modelleken is tanulmányoztuk. Ezen kívül 46 db enucleációból származó UM szövetmintán egyidejűleg tanulmányoztuk a 3-as és 4-es kromoszóma alterációit FISH-el, valamint lehetséges kapcsolatukat a minták LH-RH-R-I expressziójával.

Az mRNS expressziós eredményeink alapján az LH-RH-I receptorát sikerült detektáltuk mindkét humán kísérleti sejtvonalon, azonban az OCM-3 sejtek esetén jelentősen magasabb receptor expresszió volt megfigyelhető. Hasonlóan az *in vitro* eredményeinkhez szintén magasabb szintű LH-RH-I receptor expresszió volt kimutatható az UM xenograft *in vivo* modelleinkben is. A receptor fehérje jelenlétét sikerült igazolni immunocitokémia és Western blot technikák segítségével sejtvonalainkban, valamint Western blottal a xenograft modellekből származó szövetmintáinkban. Radioreceptor analitikai eredményeink specifikus nagy affinitású LH-RH-R-I jelenlétét mutatták mindkét kísérleti modell estén. Mind OCM-1 és OCM-3 sejtek esetén, mind a tumor szöveteken detektáltuk az LH-RH ligand jelenlétét is. Az általunk vizsgált mintákban a 4-es kromoszóma csak 14 esetben (30 %) volt normál 2 kópiában jelen, 32 esetben (70 %) a 4-es kromoszóma többlete volt kimutatható. A 3-as kromoszóma monoszómiája 16 esetben (35 %) volt megfigyelhető. 6 minta esetében (13%) 3-as kromoszóma többletet, 24 esetben (52 %) pedig normál kromoszóma számot detektáltunk. Eredményeink szerint az azonos uvealis melanoma mintákból származó 3-as és 4-es kromoszóma indexek között statisztikailag szignifikáns korreláció mutatkozott. Valamint mérsékelt különbség mutatkozott a különböző patológiai tulajdonságokkal rendelkező UM betegcsoport túlélési idejében. Az általunk vizsgált humán uvealis melanomákban azonban az LH-RH receptor expressziója független a 4-es kromoszóma megoszlásától. Eredményeink újabb információkkal szolgálnak ezen igen agresszív daganattípus genetikai hátterének megismeréséhez és a későbbiekben hozzájárulhatnak a betegség prognózisának és LH-RH receptorokon keresztüli célzott terápiájának pontosabb meghatározásához.

10 Acknowledgements

I would like to express my special thanks to my supervisor, Gábor Halmos PharmD PhD the head of the Department of Biopharmay, for his guidance throughout my PhD period, his support and his useful advice in my experimental work.

This work is dedicated to the late Andrea Treszl, PhD, who died of metastatic breast cancer. She introduced me to the world of research and inspired me to start my scientific work. Her intellectual, spiritual and personal contributions provided a great inspiration to our work in UM.

I express my gratitude to all my colleagues in the Department of Biopharmacy for creating a supportive and pleasant atmosphere.

I express my gratitude to our collaborating partners. It was a great privilege to perform scientific work with Nobel laureate Andrew V. Schally.

I am deeply grateful to my husband and my little son, Botond, for their patience, encouragement and support throughout my studies.

This work was supported by Hungarian Scientific Research Fund (OTKA) K 81596 (G.H.), TAMOP 4.2.2.A- 11/1/KONV-2012-0025 project (G.H.), TAMOP-4.2.2/B-10/1-2010-0024 (E.S), the Gedeon Richter's Talentum Foundation (E.S.), EFOP-3.6.1-16-2016-00022 (E.S) and the ÚNKP-17-3 New National Excellence Program of the Ministry of Human Capacities (E.S.). The publication is also supported by the GINOP-2.3.2-15-2016-00043 (G.H.) project. The project is co-financed by the European Union and the European Regional Development Fund. The research was also financed by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the Biotechnology thematic programme of the University of Debrecen.

11 Keywords

human uveal melanoma,

luteinizing hormone-releasing hormone receptor,

luteinizing hormone-releasing hormone ligand,

targeted cancer therapy,

aberrations of chromosome 3 and 4,

chromosome index,

fluorescence *in situ* hybridization

humán uveális melanoma

luteinizáló hormon-felszabadító hormon receptor

luteinizáló hormon-felszabadító hormon ligand

célzott daganatterápia

3-as és 4-es kromoszóma elváltozás

kromoszóma index

fluoreszcens *in situ* hibridizáció

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Candidate: Éva Sipos
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Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. **Sipos, É.**, Dobos, N., Rózsa, D., Fodor, K., Oláh, G., Szabó, Z., Székvölgyi, L., Schally, A. V., Halmos, G.: Characterization of Luteinizing hormone-releasing hormone (LH-RH-I) receptor type I as a potential molecular target in OCM-1 and OCM-3 human uveal melanoma cell lines.
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Total IF of journals (all publications): 22,646
Total IF of journals (publications related to the dissertation): 5,274

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

07 March, 2018

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Published Abstracts:

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