Yasir et al. Surgical and Experimental Pathology (2020) 3:19
https://doi.org/10.1186/s42047-020-00072-1

Surgical and Experimental Pathology

RESEARCH Open Access

Immunohistochemical expression of receptors for luteinizing hormone-releasing hormone (LHRHR) in muscle-invasive Urothelial carcinoma of urinary bladder: a potential predictive marker for targeted cytotoxic LHRH hybrid analogs

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Abstract

Background: Recurrent disease following failed chemotherapy for muscle-invasive urothelial carcinoma (UC) has no universally accepted treatment guidelines. Receptors for luteinizing hormone-releasing hormone (LHRHR) have recently been identified in urothelial cancer cell lines as well as tissue samples. These receptors can be used as target for cytotoxic hybrid analogs of LHRH. The aim of this study was to determine the frequency of LHRHR expression in muscle invasive UC by immunohistochemistry.

Methods: Fifty-two cases, including TURBTs (31) and cystectomies (21), with at least muscle invasive UC were retrieved. Of 52 patients, 41 (78.8%) were male and 11 (21.1%) were female, with age ranging from 50 to 84 years. Immunohistochemical staining for LHRHR antibody (N-20, Santa Cruz, 1:50) was performed using the LSAB method. Membranous and/or granular cytoplasmic staining was considered as a positive reaction. Scoring was based on the percentage of positive tumor cells; negative (no staining), 1+ (1–25%), 2+ (26–50%), 3+ (51–75%), 4+ (> 75%).

Results: Of 52 UC cases, 32 (61%) were AJCC stage T2, 17 (33%) were T3, and 3 (6%) were T4. Of 52 cases, 30 (58%) were positive and 22 (42%) were negative for LHRHR. Of the 30 positive cases, 16 (53%) were scored 1+, 7 (23%) 2+, 5 (17%) 3+ and 2 (7%) 4 + .

Conclusions: More than half of the cases expressed LHRHR. Two-thirds of cases demonstrated focal (< 50%) immunoreactivity, which may cause false negative results in limited tissue samples. Immunohistochemical expression of LHRHR in UC can be a predictive marker for potential efficacy of LHRH cytotoxic hybrid analogs.

Keywords: Muscle-invasive urothelial carcinoma, Luteinizing hormone-releasing hormone receptors (LHRHR), LHRH cytotoxic hybrid analogs

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Introduction

Urothelial carcinoma is one of the most common forms of malignancy in the Western world and arises from the urothelial lining of the urinary collecting systems including the ureter, renal pelvis, urinary bladder and urethra. It is the most frequent histological type of carcinoma of the urinary bladder, with approximately 81,400 new cases diagnosed by May in 2020 along with 17,980 estimated deaths in the U. S (National Cancer Institute 2020; Siegel et al. 2013). Urothelial carcinomas of the renal pelvis and ureter are relatively rare diseases and account for less than 7% of all urinary tract malignancies with 2710 new cases of these diagnosed in 2013 (Siegel et al. 2013). Seventy-five percent of patients with bladder cancer present with localized disease while 25 and 5% of patients present with regional and distant metastases, respectively. Treatment for localized disease includes Transurethral Resection Bladder Tumor (TURBT), intravesical chemotherapy, radical cystectomy, radiation therapy and neoadjuvant chemotherapy. In patients with regional and distant metastatic disease, the treatment options are chemotherapy and surgery (in selected cases). The treatment protocols for advanced/ metastatic bladder urothelial carcinoma are based on platinum based chemotherapeutic regimens with and overall response rate of 55%. Median survival after treatment is 14.8%; however the likelihood of a long term survival after 5 years ranges from 3.7 to 21.8%. Relapse of bladder urothelial carcinoma after initial chemotherapeutic application carries a poor prognosis and poses a therapeutic dilemma as there are no, universally accepted, therapeutic guidelines with a median survival of more than 7 months (Gallagher et al. 2008). New treatment modalities such as immunotherapy (including pembrolizumab, atezolizumab, nivolumab, durvalumab and avelumab) and target therapy with erdafitinib are being used as monotherapies in patients who have either failed platinum based therapy or have mutations in fibroblast growth factor 2 and 3 receptors (Nadal and Bellmunt 2019).

Luteinizing hormone-releasing hormone (LHRH) also known as Gonadotropin-Releasing Hormone (GnRH) was identified and synthesized in 1971 by one of us (A.V.S.). LHRH is a decapeptide produced by the hypothalamic neurons and secreted in a pulsatile manner into the hypophysial circulation through the pituitary portal vessels. Upon reaching the gonadotrophic cells of the anterior pituitary gland, LHRH binds to its specific receptor, also known as Type 1 LHRHR. The activation of this receptor results in the secretion of the gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from these secretory cells. In addition to the LHRH receptors found in the pituitary, several animal and human cancers have also been found to express LHRH receptors. A high incidence of LHRH receptors, for example, has been identified in human prostate cancers and prostate cancer cell lines (Halmos et al. 2000). Various investigators have shown the presence of LHRH receptors in 52% of breast cancer specimens, 80% of human ovarian cancer specimens and ovarian cancer cell lines, 80% of endometrial cancers and cancer cell lines. Recently a high incidence of LHRH receptors has been reported in non-Hodgkin's lymphoma (NHL) (Keller et al. 2005a), renal cell carcinoma (Keller et al. 2005b) and melanoma cell lines. Many of these studies have used immunohistochemical analysis to demonstrate the presence of LHRH receptor protein.

Targeted chemotherapy represents a modern oncologic strategy to improve the cytotoxic effects and decrease the peripheral toxicity of treatment. Receptors for the peptide hormones located on membrane of tumor cells can serve as targets for peptide analogs linked to cytotoxic agents. Clinical efficacy and favorable safety has been obtained in patients with LHRHR positive ovarian and endometrial cancer (Emons et al. 2014; Emons et al. 2010).

Several investigators used targeted cytotoxic LHRH analogs to assess the safety profile and therapeutic effectiveness in various human cancers expressing LHRHRs, and including prostate and endometrial cancers (Emons et al. 2014; Emons et al. 2010; Zoptarelin Doxorubicin (AEZS 108) as Second Line Therapy for Endometrial Cancer n.d.; Yu et al. 2017). Production of LHRH and LHRHR has also been reported in normal urothelial mucosa and cancer cells (Bahk et al. 2008). Although, LHRH does not induce proliferation or cell cycle changes in bladder cancer cells. However, LHRHRs on the surface of urothelial cancer cells can serve as a target for cytotoxic LHRH hybrids where the LHRH analog serves as carrier for the cytotoxic drug. Once the LHRH analog binds to the LHRHRs, the cytotoxic drug accumulates on the surface of the cancer cells and its antitumor effects are activated.

Cytotoxic hybrids of LHRH may thus enhance the therapeutic benefits of chemotherapy drugs by targeting LHRH Rs allowing selective delivery of the coupled cytotoxic molecule such as doxorubicin. Preclinical studies have shown that cytotoxic analogs of LHRH incorporating doxorubicin are far more effective than doxorubicin alone in cells expressing LHRHRs; their cytotoxic effect is minimal in cells lacking these receptors (Emons et al. 2009; Engel et al. 2012; Schally et al. 2011; Schally and Nagy 2004). Demonstration of LHRHRs in carcinoma cells is thus critical for patient selection and anticipation of a therapeutic effect. In addition, if analogs of LHRH are to be studied as second-line chemotherapeutic agents in UC, demonstrating the expression of LHRHRs in this neoplasm is critical. Therefore, the aim of this study was to evaluate the rate of expression of LHRHR in muscle invasive UC.

Methods

All patients diagnosed with UC by TURBTs or cystectomies at University of Miami Hospital from January 2009 to December 2010 were retrieved from anatomic pathology data base. Patients with in-situ carcinoma, noninvasive papillary UC and carcinoma invading up to the lamina propria (stage T1) were excluded from the analysis. In addition, patients with pure or mixed adenocarcinoma, mixed squamous cell carcinoma, small cell carcinoma, melanocytic neoplasm, hematolymphoid and mesenchymal neoplasm were also excluded. Pathology slides on the selected patients were reviewed by two GU pathologists (MJ and SY) blinded to the initial interpretation. Their diagnosis was made based on the histopathological criteria for pure UC. The neoplasms comprised of multilayered papillary projections or diffuse sheets/ nests of neoplastic cells with high nuclear cytoplasmic ratio, nuclear enlargement and atypical mitoses (Fig. 1a, b). The neoplastic cells invade the underlying lamina propria, detrusor muscle and/ or transmurally into the perivesical adipose tissue. In only few cases infiltrating carcinomas showed focal areas of squamous and spindle cell differentiation.

Immunohistochemical staining and scoring

Three micrometer histologic sections of 10% formalin-fixed, paraffin-embeded tissue were deparaffinized in xylene, rehydrated and treated with antigen retrieval solution. All slides were then treated with normal horse

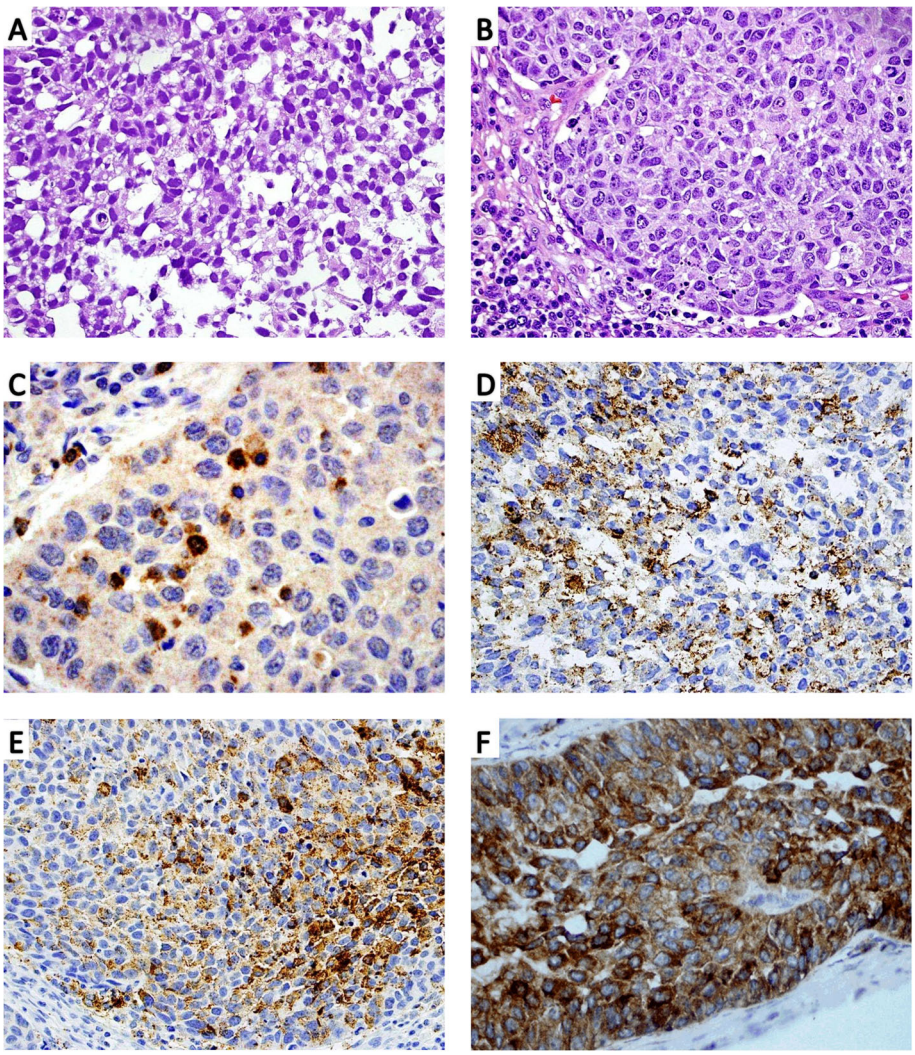

Fig. 1 Muscle invasive urothelial carcinoma. **a** Diffuse sheet of neoplastic cells with pleomorphic hyperchromatic nuclei, inconspicuous nucleoli and pale eiosinophilic cytoplasm (H&E, 20x);**b** Diffuse sheet and nest of neoplastic cells with pleomorphic vesicular nuclei, coarse nuclear chromatin, prominent nucleoli, abundant eiosinophilic cytoplasm and apoptotic debri (H&E, 20x); **c**: Focal (1+) and strong cytoplasmic granular LHRHR staining in tumor cells (LHRHR immunostain, 40x); **d:** Focal (2+) and strong cytoplasmic granular LHRHR staining in urothelial carcinoma cells (LHRHR immunostain, 20x); **e:** Strong membranous and granular cytoplasmic LHRHR immunostaining (3+) in urothelial carcinoma cells (LHRHR immunostain, 20x); **f**: Strong membranous and cytoplasmic immunostaining for LHRHR (4+) in urothelial carcinoma cells (LHRHR immunostain, 40x)

serum for 5 min and incubated with the primary antibody (LHRHR, N-20, Santa Cruz, 1:50). Slides were then incubated for 25 min with linking solution and for 25 min streptavidin-peroxidase, using phosphate-buffered saline washings between steps. Chromogenic solution was then applied. The slides were rinsed in tap water and dehydrated in increasing grades of isopropyl alcohol, cleared with xylene and mounted. Appropriate controls were stained using the same method.

Membranous and/or granular cytoplasmic staining was considered to be positive reaction. Scoring system was designed for quantitative assessment of the immunohistochemical staining using objective criteria. Scoring was based on the percentage of positive tumor cells; negative (no staining), 1+ (1 to 25%), 2+ (26 to 50%), 3+ (51 to 75%), 4+ (> 75%).

Results

Fifty-two patients with urothelial carcinoma met our study criteria. The clinicopathologic characteristics and staining profile of these cases are shown in Table 1. Thirty-one (60%) of 52 patients had TURBT and 21 (40%) patients had cystectomy. Forty one of 52 patients were male and 11 were female, with age ranging from 50 to 84 years. In the 21 patients undergoing cystectomy for UC, the mean tumor size was 3.15 cm (range: 1.5 cm to 5.5 cm). On histopathologic review, 5 (9.6%) and 1 (2%) of 52 cases showed focal squamous and sarcomatoid differentiation, respectively. One (2%) of 52 cases was classified as a nested variant of UC. Of 52 cases of invasive UC, one case had a high grade papillary urothelial carcinoma, 5 cases arose in a background of flat/ urothelial carcinoma in situ (CIS). Background lesions cannot be determined in the remaining 46 cases.

Of 52 cases, 30 (58%) were positive, and 22 (42%) were negative for LHRHR (Table 1). Of 30 LHRHR positive cases, 16 (53%) cases showed focal (score 1+) but strong immunoreactivity (Table 2, Fig. 1c). Of these 16 cases, 11 (68.5%) cases showed LHRHR positivity in approximately 10 to 25% of tumor cells. Seven (23%) of the 30 cases displayed 2+ immunoreactivity for LHRHR (Table 2, Fig. 1d). Of these 7, four, one and two showed LHRHR positivity in 30, 40 and 50% of tumor cells, respectively. Five (17%) of 30 cases showed 3+ LHRHR positivity (Table 2, Fig. 1e). Only 2 (7%) of 30 cases displayed 4+ (Table 2, Fig. 1f) positivity for LHRHR. LHRHR immunohistochemical expression was not found in the non-neoplastic adjacent urothelial mucosa.

Table 1 Characteristics of Patients with Muscle-Invasive Urothelial Carcinoma, Stratified by LHRHR Expression ($n = 52$)

Characteristic	LHRHR Expression	
	Negative (***n*** = 22)	Positive (***n*** = 30)
Age, mean, y	71.2	73.5
AJCC stage, No. (%)		
T2	13 (59)	19 (63)
T3	7 (32)	10 (33)
T4	2 (9)	1 (7)

Abbreviations: *AJCC* American Joint Committee on Cancer, *LHRHR* Luteinizing hormone–releasing hormone

Table 2 Distribution of LHRHR-Positive Urothelial Carcinoma Cases in Relation to AJCC Staging (n=30)

LHRHR			
Expression	**AJCC Stage, No. (%)**		
Score	**T2 (*n* = 19)**	**T3 (*n* = 10)**	**T4 (*n* = 1)**
1+	10 (53)	5 (50)	1 (100)
2+	5 (26)	2 (20)	0 (0)
3+	2 (11)	3 (30)	0 (0)
4+	2 (11)	0 (0)	0 (0)

Abbreviations: *AJCC* American Joint Committee on Cancer; *LHRHR* Luteinizing hormone–releasing hormone

The distribution of UC cases with positive LHRHR staining in relation to AJCC staging is shown in Table 2. Thirty-two (61.5%) of 52 UC cases were AJCC stage T2, 17 (33%) were T3, and 3 (6%) were T4. Of 32 cases with AJCC stage T2, 10 (31%) showed 1+, 5 (15%) had 2+, 2 (6%) had 3+ and 2 (6%) had 4+ LHRH staining. Thirteen (40%) of 32 AJCC stage T2 cases were negative for LHRHR. Of patients with AJCC stage T3, 5 (29%), 2 (11.7%) and 3 (17%) of 17 cases showed 1+, 2+, and 3+ LHRHR positivity respectively. Seven (41%) of these 17 cases were negative for LHRHR. Of 3 cases with AJCC stage T4, 2 (66.6%) cases were negative for LHRHR; whereas only 1 (33%) case showed 1+ LHRHR immunoreactivity. Immunohistochemistry for LHRH staining 4 + in Urothelial Carcinoma

Discussion

Patients with locally advanced or metastatic urothelial carcinoma carry a poor prognosis; treatment, after failure of cisplatin-based chemotherapy, remains a challenge. There is no universally accepted second line therapy available for patients with recurrent disease. More sophisticated strategies are required to improve therapeutic effectiveness and reduce peripheral toxicity. Targeted therapy is a modern oncologic strategy that utilizes cell membrane receptors as targets for peptide analogs linked to cytotoxic drugs (Emons et al. 2009; Engel et al. 2012; Schally et al. 2011; Schally and Nagy 2004). So far only erdafitinib has recently been proven to be the first targeted therapy (Nadal and Bellmunt 2019). Therefore, establishing the presence of LHRH receptors on the cancer cell surface is critical for predicting therapeutic response and may yield pathway to further targeted therapies in future.

Although the small sample size is a limitation of our study, but the current study is highly significant in being the first study using immunohistochemistry as a tool to demonstrate expression of LHRH receptor by urothelial carcinoma cells. Various interpretative features and limitations have also been commented upon.

Our findings indicate that more than half (30/52, [58%]) of the muscle invasive bladder urothelial carcinomas were positive for LHRHR by immunohistochemistry. Almost half of these positive cases (16 of 30) show focal (score 1+) but strong LHRHR immunoreactivity. As focal LHRHR immunostaining by the neoplastic cells may result in false negative interpretation, careful evaluation must be performed in cases with limited diagnostic material.

The remainder of the cases displayed positive staining in more than 25% of the tumor cells. All cases positive for LHRHR showed a distinct membranous and cytoplasmic/ granular staining pattern. In comparison with prior studies (Bahk et al. 2008), our data showed a lack of LHRHR immunoreactivity in adjacent non-neoplastic urothelium. A smaller study by Szepeshazi et al. (Szepeshazi et al. 2012) documented the expression of LHRHR in 18 of 18 (100%) of human bladder cancer specimens. Similar to our data, their study showed LHRHR negativity in adjacent non-neoplastic tissue.

Our study demonstrates that a high number of muscle invasive urothelial carcinomas express LHRHR by immunohistochemistry. We found that there is a quantitative variability in the staining of LHRHR in UC cells. Some variability in the intensity of LHRHR staining was also noted. However, our scoring system for LHRHR staining was primarily based on the quantitative assessment. Whether this difference in intensity and quantity of LHRHR staining has any impact on the therapeutic response must be addressed in future studies.

Conclusion

We found that the majority of the muscle invasive bladder urothelial carcinoma cases express LHRH receptors. Immunohistochemistry can be used as a tool to determine expression of LHRH receptors. Immunohistochemical expression of LHRH receptors on bladder UC cells may be a predictive marker for potential therapeutic response to cytotoxic targeted analogs of LHRH.

Abbreviations

LHRH: Luteinizing hormone-releasing hormone; LHRHR: Luteinizing hormone-releasing hormone receptors; UC: Urothelial carcinoma; TURBT: Trans urethral resection of bladder tumor; LSAB: Labeled Streptavidin–Biotin; AJCC: American Joint Committee on Cancer; GnRH: Gonadotropin-releasing hormone; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; NHL: Non-Hodgkin's lymphoma; AEZS-108: Zoptarelin doxorubicin

Acknowledgements

This study was supported by the Medical Research Service of the Veterans Affairs Department, Departments of Pathology and Medicine, the Division of Hematology/ Oncology of the Miller School of Medicine, University of Miami, the South Florida VA Foundation for Research and Education (all A.V.S) and by the L. Austin Weeks Endowment for Urologic Research (N.L.B).

Authors' contributions

All of the authors contributed in the research, data collection, analysis and manuscript writing. The author(s) read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Not applicable.

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Received: 8 May 2020 Accepted: 17 August 2020
Published online: 23 September 2020

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