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Genes coding kininogen and kinins receptors in vulvar carcinoma associated with HPV 18 infection using a novel TaqMan PCR assay

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ABSTRACT

Kinins are peptides involved in inflammatory processes, vascular permeability, proliferation and mitogenesis of tumor cells. The majority of Kinins actions are nediated through an interaction with cell surface bradykinin receptors BR1 and BR2. Kinins precursor is kininogen (kng). The changes in proteins are initiated by changes in the expression of genes coding these proteins and thus can be valuable diagnostic markers of malignant processes including vulvar carcinoma. Most of the vulvar neoplasms are planopeitheliale carcinomas growing slowly and metastasizes late. The paper presents an analysis of kininogen-kinins receptor genes expression in a 26 years old woman with rapidly progressing invasive culvar carcinoma stage II (FIGO) associated with Human Papillomarvirus 18 infection. The Patient was treated surgically by applying operation modo Way. HPV detection was performed using Consensus Primers MY09, MY 11 and type specific primers for HPV 16,18.

In RNA extracts the number of the mRNA copies for kinongen, BR1 and BR2 were assessed using QRT-PCR Taq Man.

The lack or low expression of kng mRNA, the high expression of Br1 mNRA receptor and lower expression mNRA Br2 receptor in cancer tissue was found. The elevated expression of Br1 was particularly marked in the area of the neoplastic infiltration of the tumor (BR1/BR2 44,8,BR1/kng 170).

The high BR1 expression was also shown in inguinal lymph nodes with chronic inflammation, but there was observed the kininogen gene expression too. In tissue without neoplastic cell from the surgical margin expression of all studied genes was present with predominance of kinins BR2 receptor. It was only in normal tissue from femoral lymph nodes that the predominance of kng mRNA was ascertained. The high expression of kinins receptors especially BR1 in the infiltrating carcinoma margin can be a marker of pathological significance, the proliferative potential of neoplastic cells or chronic inflammatory state in the presence of invasion carcinoma.

Key words: vulvar carcinoma, Kininogen, bradykinin receptors, QRT-PCR

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INTRODUCTION

Clinical experience indicates that tumors having similar histopathological features often vary in clinical course and response to any given treatment.

About 90% of all vulvar tumors is the squamous cell carcinoma[9,12,15] and about 85% of all vulvar carcinomas occur in women more than 40 years[1,15]. The incidence rates for invasive vulvar cancer peak among women aged 65–75 years[1,9,12].

Vulvar carcinomas is commonly indolent, grows slowly, and metastasizes fairly late. Women diagnosed with tumors larger than 2 cm were older than women with tumors smaller than 2 cm[9]. Reports of women younger than age 35 with vulvar carcinoma are unusual[5,17] and most of these patients have underlying diseases that contribute to immunosuppression[5].

The study presents the case of a 26 year old woman with invasive vulvar planoepitheliale carcinoma stage II according to FIGO in which HPV 18 infection was ascertained.

The patient was treated surgically and tissue pathology was analysed by the assessment of genes expression coding kininogen (kng) and kinin receptors BR1 and BR2.

Kinins are peptides intensively studied because of their role in inflammatory processes, vascular permeability, proliferation and mitogenesis of tumor cells what could enhance metastases[3,14,19].

Kinins are synthesized de novo at sites of tissue damage from endogenous substrate kininogen. The majority of kinins are mediated through an interaction with cell surface bradykinins receptors BR1 and BR2[19,20].

BR1 seem to be absent in normal tissue, but are synthetized de novo following tissue injury and has been shown to mediate hyperalgesia in chronic inflammation[4,16].

BR2 are widely distributed in mammalian tissue and BR2 activation causes hypotension, pain, inflammation and vascular permeability[14,16].

The observed changes in proteins connected with the kinins system are initiated by changes in the expression of genes coding these proteins; thus can be valuable diagnostic markers of malignant processes including vulvar carcinoma.

MATERIALS AND METHOD

A 26 year old woman was admitted because of the presence of tumor localized bilaterally in the region of large pudendal lips and in lips commissure. The tumor was characterized by rapid exophytic growth (during 4 month) and had 6 × 4 cm in diameter.

The patient was subjected to excisional biopsy of the labial lesion. This revealed carcinoma planoepitheliale keratodes G2. Surgical treatment was applied and the radical vulvectomy with bilateral inguinal and femoral node dissection were performed.

The tissue removed were analysed histopathologically.

At the same time, fragments of specimens from the center of the tumor, from the tumor border, surgical margin and from inguinal and femoral lymph nodes were taken for extraction of DNA and RNA.

HPV detection was performed using Consensus Primers MY09, MY11 and type specific primers for HPV 16, 18.

In RNA extracts the number of the mRNA copies for kiningen, BR1 and BR2 were assessed using QRT-PCR Taq Man.

β-Actin was included as a control of the PCR process in each run alongwith BR1, BR2 and kng. Only results obtained when β-actin levels were higher than 500 mRNA copies were taken into consideration.

The patient was invited for further examination 1 year after the surgery. Excisional biopsy specimen was taken from the operated region.

Extraction of nucleic acids. Tissue samples were incubated in lysis buffer containing 100 mmol/l. TRIS-HCl (pH 8.0), 1% SDS and 50 mg/ml proteinase K for 2 h at 55°C for RNA extraction. RNA was prepared by the guanidinium thiocyanate technique[6]. The concentration of RNA was determined by on spectrophotometric measurement using of the gene Quant-calculator in 5 μl capillaries and an assumption was that 1 OD of RNA equals 40 μg/ml. The concentration of primers was determined as 1 OD = 30.6 μg/ml.

Q-RT-PCR was performed in the reaction mixture containing (final volume 50 μl): Tth PCR Buffer, 3 mM $MgCl_2$, dNTP mix – 400 μM. of each nucleotide, Master Amp,0.5 mM $MnSo_4$, 1–10 μg of RNA Template, 0.5 μM each primers and 0.2 μM fluorogenic probe.

The following primers and probe were designed:

for mRNA of the kininogen PKNGa (5' GCACCTTCCTGGATACGCTGCATCG3' 162-186),
PKNGb (5' TGCTGTAGGCCATGAaGGAGGCGA3' 344-369),
Probe SKNG* (5' TGACTTCGTGGCCAGGGAAACCACA3' 200-225),
for mRNA of the receptor B2 PRB2a (5' GCACCTTCCTGGATACGCTGCATCG3' 44-69),
PRB2b (5' TGCTGTAGGCCATGAAGGAGGCGA3' 101-125)
and probe SRB2* (5' TCCAGCTGCCAGGACGAGCGCATCA3' 83-108),
for mRNA receptor B1 PRBla (5'GCAGGAGGCCACGAAGCCATGA3'34-56),
PRB1b(5'TGACTGGCCTCCCACGCTTGCT3'248-270),
and probe SRBl* (5'TGTGGGATCGCAGAGGGCTGGGAGA3'79-104).

The PCR mixtures were placed into a thermocycler ABI PRISM 7700 Sequence Detection System, which makes use of Perkin-Elmer 9600 thermal cycler technology and adds real-time fluorescence detection. A 488-nm argon serves as the light source, and a spectrograph interfaced to a CCD camera serves as the detector after every cycle of reaction.

The thermal conditions of reaction were as follows: RT 20 min at 60°C and PCR initial denaturation 5 min at 94°C, 40 cycles of denaturation at 94°C for 30s, hybridization of primers and probe with respect to template – at 60°C for 30s, and elongation at 72°C for 30s. In addition after the last cycle of amplification, the samples were incubated at 72°C for 10 min.

Fluorescence monitoring. The probe contains a fluorescent dye (FAM) at the 5' terminus and a fluorescent quencher (TAMRA) at the 3' terminus. When this probe is placed in the PCR, the DNA polymerase that catalyzes the PCR also cuts off any probe specifically binds to template of PCR product being generated. Cut-off results in a fluorescence increase above a large fluorescence background which was proportional to the number of DNA copies.

Analysis of PCR amplification products. Detection of amplification products was carried out using electrophoretic separation method on 6% polyacrylamide gels. After separation, the amplified DNA was stained with silver and a quality of the obtained products was assessed by comparison of the values of R_f coefficients for the separated products and the pBR332 plasmid DNA digestion with Hae III endonuclease used as marker DNA. This analysis was performed using the gel documentation system, Biotec-Fischer BaSys ID.

RESULTS

Table 1 presents results of histopathological examination of the tissue from the excised area and the number of copies of analysed genes in analysed tissue.

In this study the DNA for HPV18 was detected.

We analysed the number of mRNA BR1, BR2 and kng copies in different tissue specimens expressed per 1 μg of total RNA. Negative results of QRT-PCR were interpreted as a lack of expression of the examined genes.

The results of tissue examination one year after the surgical treatment are also presented in Table 1. It was found that macroscopic changes had occurred suggesting carcinoma recurrence during clinical examination.

TABLE 1
Results of histopathological examination of the tissue from the excised area and the number of copies of analysed genes from analysed tissue.

Nr of specimen	Specimen *histopathological result*	Number of copies mRNA/ 1μg RNA			BR1/BR2	BR1/kng	BR2/kng
		BR1	BR2	kng			
2754	Tumor center *Carcinoma planoepitheliale keratodes G2*	2 329 961	257 360	0	9	2329961	257360
2754 a	Tumor border *Infiltratio carcinomatosa diffusa profunda*	74 766	1 667	438	44.8	170	3.8
2755	Surgical margin *Lichen sclerosus*	158 902	236 794	17 777	0.67	8.9	13
2756	Inguinal lymph nodes *Lymphadenitis chronica*	2 151 292	530 158	1 282	4	1678	4.3
2757	Femoral lymph nodes *Lymphonoduli normalis*	10 985	10 435	31 274	1	0.35	0.33
3539	Biopsy specimen 1 year later *Inflammatio chronica*	1 086	0	4	1 086	271	4

DISCUSSION

Carcinoma of the vulva accounts for 3-5% of all female genital malignancies and has been increased in recent years[5,9].

In vulvar carcinoma etiology at least two mechanisms are considered the mutilation of the tumor suppressor genes (prevalently *p53*) or inactivation of tumor suppressor genes function by binding with viral proteins (E6/E7 of HPV-Human Papillomaviruses)[1,5,10,24]. HPV infections are revealed in 20%–60% of invasive cancers, especially in young age group[12].

HPV 16 appears to be a major subtype indentified in invasive carcinoma[15,20]. Most of the younger patients with vulvar carcinoma have small, invasive lesions associated with diffuse intraepithelial neoplasia[9]. On the contrary in the present case of invasive vulvar carcinoma the presence of HPV 18 by the use of specific primers was detected. The course of the tumor growth was rapid, exophytic and multifocal. The analysed patient does not have any other coexisting diseases. The profile of expression of genes coding kininogen – a substrate for kinin production and kinins receptors BR1 and BR2 was applied as biological markers of the observed pathology.

According to our data, a lack of expression for kininogen genes occurred in cancer cells from the tumour tissue. The low expression of kng gene appeared in the tumor border area, while in the tissues obtained without histopathologically confirmed cancer cells a high expression of the kininogen gene and increased expression of BR2 was observed.

Higher number of mRNA BR1 copies than mRNA BR2 in cancer tissue was also noted. The higher expression of BR1 was particularly marked in the area of the neoplastic infiltration of the tumor (BR1/BR2 44.8, BR1/kng 170). Invasion of tumor into surrounding tissues is a consequence of carcinoma cell proliferation and spread[28]. Bradykinin plays an important role in enhanced vascular permeability in tumor tissue and sustains tumor growth[26]. Our results further confirmed this observation.

In lymph nodes the positive expression of all studied genes was found. Inguinal lymph nodes involved in inflammatory process had expression patterns of mRNA BR1 and BR2 similar to that of the core tumour tissue, but the expression of kng gene was also demonstrated. Histopathologically no metastatic – carcinoma were found in the excised lymph nodes. In femoral lymph nodes the dominating *kng* gene expression was observed in the presence of balanced, lower expression of BR1 and BR2 genes. The femoral lymph nodes were histopathologically normal.

Lower expression of the kininogen gene was ascertained in cancer tissue by other researchers[18,21]. The observation of increased kinin receptor expression due to oncogenic transformation gives further importance to a mitogenic role for kinins in tumor tissue[4].

Kinins act as mitogens, stimulating DNA synthesis and thereby promoting cell proliferation[2,19]. The ability of kinins to induce cell division could enchance the spread of cancerous cells and invasion of tumor to surrounding tissues. The presence of lymph node metastases appears to be related to the size and differentiation of the tumors and the presence of infiltrating margins[23]. The BR2 has a high affinity to bradykinin and mediates most of biological processes.

BR1 have a weak affinity to intact bradykinin but possess a strong affinity to kinins metabolites (des-Arg^9-bradykinin and des-Arg^{10}-kallidin, des-Arg^{10}, Lys-bradykinin). They seem to be absent in normal mammalian tissues but appear in pathological states such as chronic inflammation and trauma[4,16] and can be upregulated by cytokines and growth factors[19].

In the study of Attanasova et al[3] the effect of bradykinin on germ cell proliferation indicate that bradykinin may be an important local factor in the regulation of spermatogonial cells proliferation and germ cell number acting via B2 receptors.

Clements et al[8] found that expression of kininogen and B2 receptor genes has been elevated at a time of extensive endometrial proliferation and tissue remodeling in midcycle. Similarly Shams et al[22] found that bradykinin is an agent in endometrium that stimulates stromal cell proliferation. The BR2 mRNA was highly expressed in proliferative and in secretory endometrium. There are also reports suggesting the potently inhibitory role of bradykinin through BR1 and BR2 in mitogenesis and proliferation in smooth muscle cells from rat mesenteric artery[11]. The inhibition occurred at a point late inprogression of the cell cycle from G1 to S phase and was dependent on the presence of kinins after exposure to PDGF (platelet derived growth factor).

Yau[27] described the role of bradykinin in the inhibition of uridine incorporation during RNA synthesis mediated by prostaglandins.

The mitogenic effect of bradykinin in cancerous lesion was ascertained in the human colon carcinoma cell lines[13]. The presence of BR2 receptors was found in squamous and adenocarcinoma of the human lung[23]. Alric et al[1] revealed the bradykinin dose-dependent inhibition of mesangial cell proliferation through the B2 receptor.

Results of our study suggests that the high expression of mRNA BR1 receptors can be a marker of vulvular pathology and mRNA BR2 is more typical of the physiological process. Data of Alric and coworkers[2] suggest that in proliferating rat mesangial cells B2 receptor stimulation is able to induce the inhibition of cell proliferation. So, the higher the expression of BR2 renders it to be a good prognostic marker, in addition to the predominance of BR1 receptors found in cancer tissue which was confirmed in this study. High expression of BR1 can indicate the intensity of cell proliferation and cancer invasion.

In our opinion the balanced expression of BR1 and BR2 in femoral lymph node specimens were a marker confirming the physiologic state opposite to the predominance of BR1 expression found in inguinal lymph nodes with chronic inflammation.

It is noteworthy that examination of this patient one year after the surgical treatment indicated no clinical or histopathological signs of cancer recurrence. Analysis of DNA isolated from the skin of the operated region shows the presence of HPV 18 virus genome. This indicate the need for frequent examination because of threat of cancer recurrence. Most recurrences are likely to be due to persisting lesions or subclinical HPV infection that had not been completely removed[7]. There was also abnormally high expression of mRNA BR1 receptors indicating chronic inflammatory state in this patient.

In summary, studies concerning the role of kinins in cell proliferation and mitogenesis are scarce when compared with those concerning inflammatory processes. Our data undoubtedly indicates that kinins play some role in neoplastic processes but further studies are required to more fully delineate the role of kininogen and kinin receptors BR1 and BR2 in cancer pathology.

REFERENCES

1. Ansink AC, Heintz APM. Epidemiology and etiology of squamous cell carcinoma of the vulva Eur J Obstet Gynecol Reprod Med. 48:111-115, 1993.

2. Alric C, Pecher C, Schanstra JP, Bascands JL, Girolami JP. Bradykinin – induced inhibition of cell proliferation and tyrosine kinase activity in rat mesangial cells. Int J Mol Med. 5:85-93, 2000.

3. Atanassova N, Kancheva L, Somlev B. Bradykinin stymulates prepubertal rat germ cell proliferation in vitro. Immunopharmacology. 40(3):173-8, 1998.

4. Bhoola K.D., Figuera C.D. Worthy K.: Bioregulation of kinins: kallikreins, kininogens and kininases. Pharmacol. Rev 44:1-80, 1992.

5. Carter J, Carlson J. Fowler J et al. Invasive vulvar tumors in young women: A disease of the immunosuppressed? Obstet Gynecol 51:307-10, 1993.

6. Chomczyński P, Sachii N. Single-step method of RNA isolation by acid guanidinum thiocyanate-phenol-chloroform extraction. Anal Biochem 162, 156-159, 1987.

7. Chua K-L, Hjerpe A. Human Papillomavirus analysis as a prognostic marker following conisation of the cervix uteri. Gynecol Oncol 66:108-113, 1997.

8. Clements J. Mukhtar A, Yan S, Holland A. Kallikreins and kinins in inflammatory – like events in the reproductive tract. Pharmacol Res. 35(6):537-40, 1997.

9. Creasman WT, Philips JL, Menck HR. The National Cancer Data Base Report on early stage invasine vulvar carcinoma. Cancer 80(3):505-513, 1997.

10. Crum C, McLachlin CM, Tate JE, Mutter GL. Pathobiology of vulvar squamous neoplasia. Cur Opinion Obstet Gynecol 9:63-69, 1997.

11. Dixon BS, Dennis MJ. Regulation of mitogenesis by kinins in arterial smooth muscle cells. Am J Physiol. 273 (1Pt1):C7-20, 1997.

12. Edwards CL, Tortolero-Luna GF, Linares AC, Malpica A, Baker VV, Cook E, Johnson E, Mithell MF. Vulvar intraepithelial neoplasia and vulvar cancer. Obstet Gynecol Clin North Am. 23:295-324, 1996.

13. Graness A, Adomeit A, Heinza R, Wetzker R, Liebmann C. A noval mitogenic signaling pathway of bradykinin in the human colon carcinoma cell line SW-480 involves sequential activation of a Gq/11 protein, phosphatidylinositol 3- kinase beta and protein kinase Cepsilon. J Biol Chem. 273(48):32016-22, 1998.

14. Hall JM. Bradykinin receptors. Gen Pharmac. 28 (1):1-6, 1997.

15. Hunter DJS. Carcinoma of the vulva: a review of 361 patients. Gynecol. Oncol 3:117-123, 1975.

16. Menke JG, Borkowski JA, Bierilo KK, MacNeil T, Derrick A, Schneck KA, Ransom RW, Strader CD, Linemeyer DL, Hess JF. Expression cloning of human B1 bradykinin receptor. J Biol Chemistry 269:21583-21586, 1994.

17. Messing MJ, Gallup DG. Carcinoma of the vulva in young women. Obstet Gynecol 86:51-54, 1995.

18. Karlsrud TS, Bur L, Aasen AO, Johansen HT. Characterisation of kininogens in human malignant ascites. Thromb. Res. 63 (6):641-50, 1991.

19. Raymond P, Decarie A, Lantin F, Raut R, Morais R, Cauture R, Adam A. A role of B1 and B2 kinins receptors in the modulation of T-kininogen during the acute phase response of inflammation. Peptides. 17(7):1163-70, 1996.

20. Regoli D, Rhaleb N-E, Drapeau G. Kinin receptor subtypes. J Cardiovasc Pharmacol. 15; S30-S38, 1990.

21. Roeise O, Sivertsen TE, Ruud TE, Bouma BN, Stadaas JO, Aasen AO. Studies on components of the contact system in patients with advanced gastrointestinal cancer. Cancer 65(6): 1355-9, 1990.

22. Shams M, Sisi P, Ahmed A. Localization of bradykinin type II receptor mRNA in human endometrium. Mol. Hum. Reprod. 2(1):35-9, 1996.

23. Trifilieff A, Lach E, Dumont P, Gies JP. Bradykinin binding sites in healthy and carcinomatous human lung. Br J Pharmacol 111(4):1228-32, 1994.

24. Trimble CL, Hildesheim A, Brinton LA, Shah KV, Kurman RJ. Heterogenous etiology of squamous carcinoma of the vulva. Obstet Gynecol 87:59-64, 1996.

25. Welkoborsky HJ, Dienes HP, Hinni M, Mann WJ. Predicting recurrences and survival in patients with laryngeal cancer by means of DNA cytometry, tumor front grading, and proliferation markers. Ann Otol Rhinol Laryngol 104:03-509, 1995.

26. Wu J, Akaike T, Maeda H. Modulation of enhanced vascular permeability in tumors be a bradykinin antagonist, a cyclooxygenase inhibitor, and a nitric oxide scavenger. Cancer Res. 58(1):159-65, 1998.

27. Yau L, Pinsk M, Zahradka P. Inhibition of RNA synthesis by bradykinin involves both the B1 and B2 receptors subtypes. Arch. Biochem. Biophys. 328 (1):115-121, 1996.

28. Yilmaz T, Hosal AS, Gedikoglu G, Kaya S. Prognostic significance of histopathological parameters in cancer of the larynx. Eur Arch Otorhinolaryngol 256:139-144, 1999.