Published in final edited form as:

Pathol Res Pract. 2016 April ; 212(4): 340–344. doi:10.1016/j.prp.2015.11.017.

Inner nuclear membrane localization of epidermal growth factor receptor (EGFR) in spontaneous canine model of invasive micropapillary carcinoma of the mammary gland

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Abstract

The epidermal growth factor receptor (EGFR) has been described in the nucleus of primary tumors. Accumulation of EGFR at the nucleus is linked to DNA synthesis and cell proliferation, but the pathological significance of nuclear EGFR is not completely understood. The aim of this study was to investigate the nuclear localization of EGFR in invasive micropapillary carcinoma (IMPC) that is an aggressive neoplasm of canine mammary gland. Confocal immunofluorescence of formalin and paraffin-embedded tissue was used to access the subcellular localization of EGFR. Our results demonstrated that EGFR co-localizes with the inner nuclear envelope marker, Lamin B1 in IMPC. Furthermore, EGFR was not localized within the nucleus or at the inner nuclear envelope membrane in mammary carcinoma in mixed tumor (CMT) that is associated with a better prognosis than other malignant histological types. This finding could be useful as a predictive biomarker of therapeutic response for IMPC.

Keywords

Invasive micropapillary carcinoma; nuclear EGFR; confocal microscopy

Introduction

Invasive micropapillary carcinoma (IMPC) of the breast, in human species, is a morphologically distinct, and rarely observed, form of invasive ductal carcinoma that

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Conflict of interest: We declare that we have no conflict of interest.

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presents lymphotropism and aggressive behavior [26]. Previous work, evaluating canine IMPC demonstrated several similarities to the human counterpart, for example, a high incidence of lymph node metastasis, high histological grade, and low overall survival rates were observed [4, 6, 12-14].

The nuclear EGFR is involved in many cellular processes that are important in cancer progression [22, 23]. Our group has shown previously that the nuclear EGFR translocation is a clathrin-dependent pathway in a cancer cell line [8]. However, the nuclear EGFR function is continuing to be elucidated and appears that the nuclear EGFR plays an important role in cancer development and progression.

Nuclear EGFR have been found to be related with poor prognosis in several cancer types, such as breast cancer, esophageal squamous cell carcinoma, oropharyngeal and ovarian cancers [19, 24, 29, 39]. The aim of this work was to evaluate the nuclear localization of EGFR in canine mammary IMPC. Here, we show that EGFR is found within the nucleus of IMPC. Furthermore, EGFR is co-localized with a nuclear inner membrane marker, Lamin B1. The EGFR nuclear accumulation was not observed in mammary CMT, a less aggressive type of carcinoma. Taken together, these results evaluate for the first time a nuclear EGFR localization in a spontaneous canine model of IMPC of the mammary gland.

Material and Methods

Case Selection, Histopathological Analysis and Overall Survival

Five cases of IMPC and six cases of CMT of canine mammary gland were selected at Laboratory of Comparative Pathology of the Institute of Biological Sciences at “Universidade Federal de Minas Gerais”.

Primary tumor specimens were fixed in 10% neutral buffered formalin and paraffin-embedded (FFPE), and 4µm thick histological sections were cut and stained with haematoxylin and eosin. All cases were reviewed and re-classified independently by two veterinary pathologists (COG and GDC). In brief, carcinomas with cystic formations containing nests of epithelial cells with a moruliform appearance (infiltrating micropapillary pattern), associated or not with ‘in situ’ micropapillary areas, were diagnosed as IMPC [5] (Figure 1A and 1B). The morphological criteria used for the diagnosis of the CMT lesions were proposed by Misdorp *et al.* [37]. Inclusion criteria were the integrity of paraffin blocks from each case prior diagnosis of CMT. The invasive areas of IMPC and CMT were graded according to the Nottingham grading system [9, 10]. Overall survival time was defined (in days) as the period between surgery and death because of the tumour. The follow-up period was 308 days for IMPC and 909 days for CMT cases. Kaplan–Meier method was applied and statistical significance was examined using a log-rank test. A probability values below 0.05 were considered significant.

Confocal microscopy

Immunofluorescence was performed as previously described with minor modifications [15, 17, 32, 33]. In Brief, FFPE tissue sections were dewaxed, rehydrated and unmasked in trilogy solution (Cell Marque, Koclin, CA, USA) in pressurized heating (125°C) during 20

minutes according to manufacturer's instructions. Next, samples were rinsed in Phosphate Buffered Saline (PBS, 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution, pH 7.4) (Sigma-Aldrich, Carlsbad, CA, USA), and then incubated in PBS containing 0.2% Triton X-100 (Sigma-Aldrich) for another 20 minutes and blocked in PBS containing 1% Bovine Serum Albumin (BSA, Sigma-Aldrich) for 30 minutes. The sections were next incubated with a mouse monoclonal antibody against EGFR which interacts with the extracellular domain of this receptor (1:100, clone 31G7, Carlsbad, Zymed - Life Technologies, Carlsbad, California, USA) a rabbit polyclonal antibody against the nuclear envelope marker Lamin B1 (1:1000, Abcam, Cambridge, MA, USA), and to evaluate a plasma membrane localization a mouse monoclonal antibody against E-cadherin was used (1:80, clone 4A2C7, Life Technologies) overnight at 4°C, then rinsed 3 times for 5 minutes in PBS. Subsequently, sections were incubated with Alexa Fluor® 488 Goat Anti-Rabbit IgG antibody (1:1000, Life Technologies), Alexa Fluor® 555 Goat anti-mouse IgG antibody (1:1000, Life Technologies) and, Hoechst 33258 (1µg/mL, Life Technologies) for 1 hour at room temperature. Next, samples were washed 3× in PBS for 10 minutes and then mounted in Prolong Gold Antifade reagent (Life Technologies). The negative control was included in all reactions by omitting primary antibodies. Images were collected using a Zeiss LSM 5 Live or 880 (Carl Zeiss, Jena, Germany) confocal microscopes using an oil 40× 1.3 NA or an oil 63× 1.4 NA objective lens. Samples were excited at: 405 nm and observed at 415-480 nm to detect Hoechst, 488 nm and observed at 500-525 nm to detect Alexa Fluor 488. 532 (LSM 5 Live) or 543 (LSM 880) nm and observed using a 560 Long pass filter for LSM 5 Live and similar configuration using GaAsP detector for LSM 880 to detect Alexa Fluor 555 signal. The software Zeiss Efficient Navigation (ZEN) was used for orthogonal projections (XY, XZ, YZ). The fluorescence microscopy results were evaluated in 10 invasive areas of IMPC and were collected 10 images from each IMPC. The fluorescence microscopy results were evaluated in 5 invasive areas of CMT and were collected 5 images from each CMT.

Western blot analysis

To evaluate the anti-EGFR antibody specificity in canine specie, western blot analysis was performed using two fresh samples of canine mammary tissues, as previously described with minor modifications [13, 17, 32, 33]. The two samples consisted in one normal mammary gland tissue sample and one carcinoma mammary gland sample. Previous study demonstrated that SK-Hep-1 cells, human liver adenocarcinoma, overexpress EGFR and It was used as a positive control [8]. Whole-cell extracts were obtained from homogenised samples by using a lysis buffer [1% Triton X-100; 100mM Tris/HCl, pH 8.0; 10% glycerol; 5mM EDTA; 200mM NaCl; 1 mM DTT and 1% of protease and phosphatase inhibitors cocktails (Sigma-Aldrich)]. Lysates were centrifuged at 13,000 *g* for 10 min at 4 °C and quantified using the Bradford assay reagent from Sigma-Aldrich. Extracts of protein (60µg) was separated by electrophoresis on a denaturing 8% polyacrylamide–sodium dodecyl sulphate gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 hour in Tris-buffered saline (TBS) containing 5% (w/v) nonfat dry milk and 0.05% Tween-20 and incubated with specific anti-EGFR mouse monoclonal antibody (1:100, clone 31G7, Zymed - Life Technologies) in TBS containing 5% (w/v) nonfat dry milk and 0.05% Tween-20. As a loading control, the membranes were reprobated with anti-GAPDH (1:2000; Santa Cruz, TX, USA). After

washing, the membranes were incubated with a horseradish peroxidase-conjugated anti-mouse secondary antibody (1:2000; Sigma Aldrich). Immunoreactive bands were visualized using an enhanced chemiluminescence reagent (ECL Plus; GE Healthcare Life Sciences, Piscataway, NJ, USA).

Results

The age of animals ranged from 7 to 13 years (mean 10.5 ± 2.30) at the time of surgery, and most were crossbreed (3/4, 75%). Tumours larger than 5 cm were predominant (3/4, 75%) and regional metastasis was observed in 100% of the dogs (4/4). Regarding the IMPC histopathological analysis, a histological grade I, II and III was observed in 40%, 40% and 20% of the samples, respectively. Concerning overall survival, all five dogs died because of mammary neoplasia and the overall survival time ranged from 30 to 308 days (median 120 days). The clinicopathological and survival findings of the canines are presented in Table 1.

We performed confocal microscopy to observe the nuclear localization of the EGFR in invasive areas of IMPC. First, we performed double-labeling of EGFR with Lamin B1 in all micropapillary cases. The Lamin B1 was used as a marker of the inner nuclear membrane (INM), since we wanted to evaluate EGFR nuclear localization. Our results showed that all 5 cases of IMPC tested, EGFR is co-localized with Lamin B1 (Figure 2A). This pattern was observed predominantly in the INM. Three-dimensional reconstruction of serial confocal immunofluorescence images confirmed that EGFR co-localizes with Lamin B1 (see white arrows) (Figure 2B). We also performed confocal immunofluorescence for E-cadherin and Lamin B1 to demonstrate the expected subcellular localization of a plasma membrane protein in invasive areas of IMPC of canine mammary gland (Supplementary Figure 1).

Furthermore, EGFR staining was negative in normal canine mammary gland and these samples were used as a negative control (Supplementary Figure 2). The western blot analysis was performed to show the EGFR antibody specificity in canine species. SK-Hep-1 cells were used as a positive control (human liver adenocarcinoma) (Supplementary Figure 3). The EGFR antibody detected a 170 kDa band in all samples, which is expected size of the receptor. The EGFR localization was also investigated in CMT that correspond to a less aggressive type of carcinoma in dogs [5, 7]. We have selected six cases of CMT (clinicopathological and survival data are shown in Table 2) that showed better overall survival than IMPC (IMPC, median 120 days; CMT, did not reach median survival; $p=0.0017$) (Graph 1.). We performed confocal microscopy to observe the EGFR localization in invasive areas of CMT. First, double-labeling of EGFR with Lamin B1 was performed in all 6 CMT cases. Our results showed that none of the cases presented a nuclear EGFR localization, different from what was observed in the IMPC (Supplementary Figure 4).

Discussion

A number of receptor tyrosine kinases (RTK) have been described in the nucleus, such as, hepatocyte growth factor receptor (HGFR/c-Met), fibroblast growth factor receptor (FGFR), insulin receptor and, the four members of the epidermal growth factor receptor family (EGFR) [17, 22, 27, 30, 32]. Nuclear EGFR has been detected in several cancer types [19,

22, 23], but this study is the first to demonstrate the nuclear localization of EGFR in IMPC from canine mammary gland.

There is little information about the route by which receptors are trafficked from the cell surface to the endoplasmic reticulum (ER) and then to the nucleus, but precedent exists with both viruses and toxins [2, 25, 34]. Few studies demonstrated that RTKs could travel from plasma membrane to nucleus. For example, biotinylated HGFR at the cell surface, prior to stimulation with HGF, could be recovered in the nucleus after cells were stimulated [17]. De Angelis *et al.* using time lapse confocal microscopy showed that fluorescent labeled EGF reached the nucleus after ~ 10 minutes of stimulation. Furthermore, they demonstrated the first endocytic steps for EGFR nuclear translocation is a dynamin and clathrin-dependent pathway [8]. There are two hypotheses of the route of EGFR from ER to the nucleus. The first, suggest that EGFR at ER interact with the Sec61 translocon that then mediated receptor retrotranslocation to the cytoplasm. Further, EGFR binds to HSP 70 to maintain the receptor in a soluble state following extraction from the ER membrane and subsequent association with importin- β leads to nuclear import. We speculate that the small fraction of EGFR that is dispersed at the chromatin could represent the very small fraction of the receptor that can translocate to the nucleus by this pathway in IMPC samples [3, 21]. In mammals, the membrane system of the nuclear envelope (NE) is contiguous with ER. The NE is a double membrane structure punctuated by pores where the two membranes are joined [35]. Wang and colleagues proposed a mechanism called integral trafficking from ER to the NE transport (INTERNET) to refer to the inner nuclear membrane (INM) targeting process for the ER-to-NE transport of EGFR [38]. In this work, we show that nuclear EGFR in IMPC mainly co-localizes with the INM marker Lamin B1. Thus, The INTERNET pathway could explain or be involved in the nuclear translocation of this receptor. Interestingly, proteins of the INM are likely to play major roles in the dynamics and structure of the chromatin and lamina [11, 16, 18]. During late prophase occurs disassemble of nuclear membranes and protein phosphorylation is implicated in this process [11]. Further studies will be necessary to demonstrate if nuclear EGFR is involved with phosphorylation of lamins and chromatin organization.

In addition, nuclear EGFR expression has been shown as a novel prognostic indicator for poor survival in patients with various human tumor tissues including cancers of the esophagus, skin, breast, mouth, oropharynx, lung and ovary [19, 20, 24, 28, 29, 36, 39]. Here, we explored only the endogenous nuclear localization of EGFR in IMPC. Cassali *et al* [4, 6] showed that canine IMPC is an important comparative model to the human IMPC counterpart and indicated that the morphological and immunohistochemical features of the canine model is similar to what is observed in humans [4, 6, 12, 14]. The five cases of IMPC evaluated in our study presented poor prognosis, high rates of lymph nodes metastasis, high tumor size and poor overall survival [12-14]. The EGFR is not present within the nucleus or in the INM in CMT, which has better prognosis than other malignant histological types [5, 7]. Bertagnolli, *et al*, 2011, and Ribeiro, *et al*, 2012, shown using histopathological analyses that in invasive areas of CMT the EGFR expression is decreased [1, 31]. The 6 cases of CMT evaluated in our study presented better prognosis, no lymph nodes metastasis and high overall survival. Therefore, this study maybe can give a new window in terms of the importance of nuclear localization of EGFR in IMPC.

Conclusion

This study is the first to demonstrate the nuclear localization of EGFR in the canine spontaneous model of IMPC of the mammary gland. This finding could be related to lymphotropic and metastatic behavior of this neoplasm in canine species. To better understanding, the importance of nuclear EGFR in the progression and poor prognosis of IMPC a high number of cases should be evaluated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant 1R03TW008709 and grants from CNPq, FAPEMIG and CAPES. The microscopic data shown in this work was obtained using the microscopes of “*Centro de Aquisição e Processamento de Imagens*” (CAPI-ICB/UFGM). We thank Karine Araujo Damasceno for providing the clinicopathological and survival findings of CMT used in this study.

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Figure 1. Histopathological appearance of the spontaneous canine model of invasive micropapillary carcinoma

A, Invasive micropapillary areas characterised by neoplastic epithelial cells within cystic spaces (arrows). HE stain. Scale bar = 60 µm. **B**, An invasive area may be observed in detail. HE stain. Scale bar = 30 µm.

Figure 2. Nuclear expression of EGFR in spontaneous canine model of invasive micropapillary carcinoma

A, Confocal immunofluorescence images of the nuclear envelope marker Lamin B1 (green), EGFR (red) and the nucleus was stained with Hoechst (blue). Note that EGFR is co-localized with Lamin B1. The images confirm the localization of EGFR in the inner nuclear membrane of invasive micropapillary carcinoma. The merged image demonstrates that EGFR co-localizes with Lamin B1 (see white arrows in B). **B**, Crop image of serial optical sections collected for three-dimensional reconstruction; x-z sections are shown at the top, and y-z sections are shown at the right of each image. White arrows demonstrate that EGFR co-localizes with Lamin B1. Images are representative of what was observed in n=5 separate primary tumor cases and were collected 10 images from invasive areas of each case. Scale Bar = 20 μ m.

Graph 1. Kaplan-Meier survival curve

Kaplan-Meier survival curve for the animals according to the histologic types: IMPC (invasive micropapillary carcinoma; n = 5; median, 120 days); CMT (carcinoma in mixed tumor; n = 5; did not reach median survival); (p=0.0017).

Table 1

Clinicopathological and survival findings of IMPCs of the canine mammary gland.

	Age (years)	Breed	Tumor Size (cm)	Lymph node Metastasis	Histological Grade*	Overall survival (days)#
Case 1	13	NA	NA	NA	I	30
Case 2	12	Labrador	7.5	Yes	II	60
Case 3	11	Crossbreed	3	Yes	III	120
Case 4	7	Crossbreed	9.2	Yes	I	120
Case 5	10	Crossbreed	10	Yes	II	308

NA, Not Available.

*Histological grading based on that of Elston and Ellis (1991, 1998) [9, 10].

#Overall survival time was defined as the period (in days) between surgery and death due to the tumor.

All canines evaluated died due to the disease.

Table 2

Clinicopathological and survival findings of CMT of the canine mammary gland.

	Age (years)	Breed	Tumor Size (cm)	Lymph node Metastasis	Histological Grade*	Overall survival (days)#
Case 1	14	Peckinese	3	No	I	1400
Case 2	10	Poodle	0.5	No	I	909
Case 3	11	NA	4	No	II	1060
Case 4	10	NA	3	No	I	1375
Case 5	7	Poodle	4	No	II	1238
Case 6	14	SRD	3.5	No	I	618

NA, Not Available.

*Histological grading based on that of Elston and Ellis (1991, 1998) [9, 10].

#Overall survival time was defined as the period (in days) between surgery and death due to the tumor.

Only in the case 1, the bitch died due to the disease. In the cases, 2, 3 and 4 the dogs were censured. For cases 5 and 6, canines were alive on the last contact.