Gynecologic Oncology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Expression of DNA damage response proteins in cervical cancer patients treated with radical chemoradiotherapy

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HIGHLIGHTS

- DDR proteins were measured in cervical cancer patients treated with CRT.
- ATM, PARP-1, DNA-PKcs, Ku70 and Ku86 were quantified using fluorescence IHC.
- Reduced DDR protein expression was associated with worse 5-year outcomes.

ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form 23 December 2016
Accepted 28 December 2016
Available online xxxx

Keywords:
Cervical cancer
Chemoradiotherapy
ATM
PARP-1
DNA-PKcs

ABSTRACT

Objective. The management of locally advanced cervical cancer has improved significantly with the advent of cisplatin-based chemoradiotherapy (CRT) as the primary treatment regimen. Nevertheless, a significant proportion of patients fail to respond or relapse on this treatment and have a very poor prognosis. Our goal was to determine the prognostic value of a panel of proteins involved in detection and repair of DNA damage.

Methods. We performed fluorescence immunohistochemistry, and used software analysis to assess expression of DNA damage response proteins ATM, DNA-PKcs, PARP-1, Ku70 and Ku86 in 117 pre-treatment specimens from patients with locally advanced cervical cancer. We compared expression to clinicopathologic correlates to determine prognostic significance.

Results. Five-year progression-free survival was significantly lower in the low expressors than in high expressors of ATM (35% vs. 58%, $p = 0.044$) and PARP-1 (24% vs. 61%, $p = 0.003$), and showed a trend to significance for DNA-PKcs (30% vs. 60%, $p = 0.050$). Low expression of the same proteins also correlated significantly with lower overall survival. In multivariable analysis, adjusted for FIGO stage and tumor size, low ATM and PARP-1 expression was significantly associated with both poorer progression-free and overall survival. Pairwise analyses indicated that expression levels of these proteins were correlated.

Conclusions. Expression of DNA damage response proteins in cervical cancer is associated with outcome in patients treated with CRT. Immunohistochemical analysis of these proteins may be useful in guiding treatment decisions in such patients.

1. Introduction

Cervical cancer is among the most commonly diagnosed cancers in women, with over half a million new cases worldwide annually resulting in over 270,000 deaths [1]. A significant proportion of patients are diagnosed with locally advanced cervical cancer, and are often treated with cisplatin-based chemoradiotherapy (CRT). Although this synergistic use of chemotherapy and ionizing radiation has significantly improved local control and overall survival [2], approximately 40% of patients succumb to disease within five years [3]. These dismal outcomes have prompted research efforts to identify molecular characteristics of tumors which predict their resistance to CRT, or which suggest the utility of novel targeted therapies.

Tumor cell cytotoxicity from CRT generally correlates with the level of sustained DNA damage induced by this treatment regimen. Accordingly, a major mechanism by which cancer cells exhibit radioresistance

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http://dx.doi.org/10.1016/j.ygyno.2016.12.025
0090-8258/

is through an increase in their DNA repair capacity [4]. Radiation induces several types of DNA damage, of which double-strand DNA breaks (DSBs) are the most lethal [5]. The cellular DNA damage response (DDR) is a complex signal transduction pathway responsible for detection and the influence of cellular responses to DNA damage [6]. This signaling network includes proteins that detect DNA lesions, and activate signaling pathways to halt cell cycle progression. Key signaling proteins include ataxia telangiectasia mutated (ATM) and ATM-and Rad3-related (ATR). IR-induced DSBs are repaired by one of two major repair pathways: the non-homologous end joining (NHEJ) pathway, or homologous recombination repair (HRR). The NHEJ pathway is particularly error-prone [7], as it involves direct ligation of broken DNA strands with minimal corrective editing. It requires the proteins Ku70, Ku86 and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), and their deficiency leads to radiosensitization of the affected cells [8]. An understanding of the mechanisms of DNA repair has guided development of therapeutic agents such as poly (ADP-ribose) polymerase (PARP) inhibitors, which preferentially kill tumor cells in a process known as "synthetic lethality" [8]. This term refers to the selective vulnerability of tumor cells to PARP inhibition when they are genetically deficient in the breast cancer susceptibility genes 1 and 2 (BRCA1/2), ATM or ATR [9–11]. Effective deployment of such therapies requires knowledge of the expression levels of proteins that regulate the damage sensing, DNA repair and signaling processes. A better understanding of interactions between different DDR pathways will provide further therapeutic opportunities [6].

Cervical cancer exhibits significant genomic instability and high mutation rates [12–14], in part due to persistent infection with human papillomavirus (HPV) and inactivation of critical tumor suppressors [15,16]. There is very little information about the pre-treatment expression of DDR proteins in cervical cancer, especially in locally advanced cancer treated with CRT. While some studies have evaluated expression of Ku70, Ku86 and DNA-PKcs proteins in patients treated primarily with radiotherapy [17–19], the reported associations between expression and prognosis have varied, in part due to differences in methodology among studies. Furthermore, whereas radiation causes DNA damage that is repaired primarily by NHEJ, cisplatin creates inter- and intra-strand adducts that are repaired by nucleotide excision repair, HR and/or the Fanconi Anemia pathway [20–21]. The complex interplay of pathways involved in DNA repair following CRT is thus not captured in studies focused on radiotherapy alone. To our knowledge, no prior studies have comprehensively assessed expression of the key proteins comprising the DDR response, nor whether CRT-sensitive or -resistant tumors are characterized before treatment by a specific pattern of expression of these proteins. This information may be important because, just as expression of mismatch repair proteins is highly relevant to microsatellite unstable cancers [22], expression of DDR proteins may be a key characteristic of cervical cancer. The prognostic value of these proteins either alone or in combination would significantly improve the management of patients with locally advanced disease.

The aim of this study was to determine if expression of key DDR proteins is associated with survival in women with locally advanced cervical cancer treated with CRT. We used fluorescence immunohistochemistry and software analysis to detect and quantify ATM, PARP-1, DNA-PKcs, Ku70 and Ku86 in treatment-naïve patient biopsy specimens. Our overarching goal was to determine if these proteins could serve as useful biomarkers for response to CRT.

2. Methods

2.1. Patient selection and chart review

After receiving ethics approval from the local institutional research board, we identified patients with locally advanced cervical cancer who were treated with radical CRT, with curative intent, at a single institution between 1999 and 2008 (Tom Baker Cancer Centre, Calgary, Alberta, Canada). Standard pre-treatment staging procedures included history, physical examination under anesthesia with cystoscopy and sigmoidoscopy, chest X-ray, and CT of the abdomen and pelvis to determine nodal status prior to treatment. Patient inclusion criteria were the following: a) completion of the planned CRT within 180 days of diagnosis, b) FIGO stages IB to IVA. Clinical and treatment details were extracted from the patient charts.

2.2. Treatment

Radiotherapy was administered as a combination of whole pelvis external beam radiotherapy (EBRT) and low-dose-rate or high-dose-rate brachytherapy, per our center's policy. Pelvic EBRT was delivered using a four-field technique and high-energy photons (most commonly 18 MV). The most common radiotherapy dose and fractionation regimen was 45 Gy in 25 equal fractions over 5 weeks. Treatment was administered as one fraction daily, five days a week. Weekly cisplatin chemotherapy at 40 mg/m^2 was administered concurrent with EBRT. After treatment, patients were monitored with routine clinical examinations, typically every three months for the first year, every four months for the second year, then every 6 months for up to 5 years. Follow-up imaging was performed as clinically indicated.

2.3. Fluorescence immunohistochemistry

Tissue microarrays (TMAs) were constructed from formalin-fixed, paraffin-embedded (FFPE) pre-treatment biopsies, and fluorescence immunohistochemistry was performed on 4 μm sections as described previously [23]. Heat-induced epitope retrieval was performed using a decloaking chamber (Biocare Medical, Concord, CA, USA) for PARP-1, Ku70 and Ku86 by heating slides to 121 °C for 6 min, in a citrate-based (pH 6.0) target retrieval solution (S1699, DAKO, Mississauga, Canada). For ATM and DNA-PKcs, retrieval was completed at 121 °C for 3 min in citrate-based or Tris/EDTA-based (pH 9.0) target retrieval solutions (S2367, DAKO), respectively. The following primary antibodies were used: ATM (rabbit monoclonal, clone Y170, 1:1000, Epitomics, Burlingame, CA, USA), PARP-1 (rabbit monoclonal, clone E102, 1:5000, Epitomics), vimentin (mouse monoclonal, clone V9, 1:2000, DAKO), DNA-PKcs (rabbit monoclonal, clone Y393, 1:400, Abcam, Cambridge, MA, USA), Ku70 (rabbit polyclonal [H308], 1:1000, Santa Cruz, Dallas, TX, USA), and Ku86 (mouse monoclonal, clone B-1, 1:1000, Santa Cruz). All antibodies were diluted with SignalStain protein blocking reagent, and incubations were performed at room temperature for either 30 (Ku70 and Ku86) or 60 min (ATM, PARP-1, and DNA-PKcs), along with a pan-cytokeratin antibody (guinea pig polyclonal, catalog number BP5069, 1:100, Acris, San Diego, CA, USA) to identify tumor epithelia. Where appropriate, primary antibodies were followed with anti-rabbit or anti-mouse EnVision + (K4011 or K4007, DAKO) secondary antibodies, and visualized with TSA-Plus Cy3 or Cy5 signal amplification reagents (Perkin Elmer, Waltham, MA, USA). The pan-cytokeratin was followed with an Alexa 488-conjugated secondary antibody (A11073, 1:200, Thermo Scientific, Burlington, ON, Canada). After immunostaining, slides were coverslipped using ProLong Diamond anti-fade mounting medium with DAPI to identify nuclei (P36965, Thermo Scientific), and stored at 4 °C until scanned.

2.4. Image acquisition and analysis

Automated image acquisition was performed using the Aperio Scanscope® FL (Aperio Inc., Vista, CA, USA) slide scanner. Images were then analyzed using the AQUAnalysis® program, version 2.4.4.1 as described [23]. Briefly, the software calculated the mean fluorescent pixel intensity within each compartment defined (tumor as the cytokeratin-positive tissue area, and stroma as the vimentin-positive area), and reported these data as AQUA scores. These compartment-specific scores were determined for each TMA spot. The mean AQUA

Please cite this article as: C.K. Ho, et al., Expression of DNA damage response proteins in cervical cancer patients treated with radical chemoradiotherapy, Gynecol Oncol (2017), http://dx.doi.org/10.1016/j.ygyno.2016.12.025