Role of Cancer Stem Cell, Apoptotic Factor, DNA Repair, and Telomerase Toward Radiation Therapy Response in Stage IIIB Cervical Cancer

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ABSTRACT

Objectives: Cancer stem cells are involved in radioresistant cancers. Transcription factors Sry-related HMG box (SOX2) and octamer binding transcription factor 4 (OCT4) can confer pluripotent cell characteristics and self-renewal ability and are involved in carcinogenesis, metastasis, tumor recurrence, and resistance to therapy. Apoptosis, DNA repair, and telomerase factors also contribute to radioresistance. We sought to identify the role of SOX2 and OCT4 as cancer stem cell markers and their effects on apoptosis (via caspase 3), DNA repair (Chk1) and telomerase (hTERT) in conferring resistance to radiotherapy. *Methods*: We conducted a case-control study of 40 patients with stage IIIB cervical squamous cell carcinoma who completed radiation therapy at Cipto Mangunkusumo Hospital, Jakarta, Indonesia. The patients were classified according to their treatment response as having exhibited a complete or incomplete response. Clinical follow-up and Pap smears were performed between six and 12 months after therapy for those with a good initial response to determine the final response to therapy. Immunohistochemistry was used to analyze SOX2, OCT4, caspase-3, Chk1, and hTERT expression in paraffin sections of the initial biopsy. *Results:* Strong expression of SOX2 ($p = 0.011$, $p = 0.001$) and OCT4 ($p < 0.001$, $p < 0.001$) was significantly associated with both an incomplete initial and final therapy response, respectively. Multivariate analysis showed that SOX2 and OCT4 expression levels were the strongest markers of an incomplete response to radiotherapy (odds ratio (OR) = 5.12, $p = 0.034$, and OR = 17.03, $p = 0.004$, respectively). *Conclusions:* Strong expression of SOX2 and OCT4 may be a good indicator of incomplete radiotherapy outcome in patients with stage IIIB cervical cancer.

Cervical cancer is one of the most common cancers found in women worldwide, especially in developing countries such as Indonesia. The 2012 GLOBOCAN data show that cervical cancer was the fourth most common cancer in women after breast, colorectal, and lung cancers. These data include an estimated 528 000 new cases and 266 000 deaths, and a five-year-prevalence of 1 547 161 cases.[1] The cancer registry of Himpunan Onkologi Ginekologi Indonesia reported 1474 cervical cases in 2013; 67.7% were categorized as locally advanced stage cancer (stage IIB–IVA), and 39.1% were stage IIIB cancer.[2]

The primary choice of treatment for advanced stage cervical cancer is radiation, but radioresistance has recently become a problem. The rates of treatment failure for post pelvic radiation of cervical cancer are

about 10% for stage IB, 17% for stage IIA, 23% for stage IIB, 42% for stage III, and 74% for stage IVA. The rates of recurrence are 58% at one year and 7% at two years.[3]

Research has identified the characteristics of stem cells found in cancer tissue, which are referred to as cancer stem cells (CSCs). CSCs represent a special subpopulation found in tumor tissue that exhibits the potential for self-renewal and pluripotency. CSCs are thought to be responsible for tumorigenesis, metastasis, resistance to therapy, and tumor recurrence.[4–7] Markers of CSCs include Sry-related HMG box (SOX2) and octamer binding transcription factor 4 (OCT4), which are thought to regulate the apoptosis pathway (via caspase-3), telomerase function (via human telomerase reverse transcription or hTERT), and DNA damage repair (via checkpoint kinase 1 or Chk1).[8–11]

The purpose of this study was to assess these markers and determine whether their expression levels provide clinical information useful for predicting the response to radiotherapy.

METHODS

This case-control study focused on the role of CSCs in stage IIIB cervical cancer and the response to treatment (radiation). This study was performed in the oncology clinic of the Obstetrics and Gynecology Department, Radiotherapy Department, and Pathological Anatomy Department of Faculty of Medicine Universitas Indonesia/Cipto Mangunkusumo Hospital from September 2015 to November 2016. Forty-eight patients were included. The inclusion criteria were patients with stage IIIB cervical cancer diagnosed according to the International Federation of Obstetrics and Gynecology criteria and histopathologically proven, completion of radiotherapy or chemoradiotherapy, adequate paraffin blocks, and follow-up by physical examination and Pap smear to monitor cancer recurrence for a minimum of six months and a maximum of 12 months after therapy (total sampling). Patients with a double primary tumor, inaccessible data, or inadequate paraffin blocks for immunohistochemical analysis were excluded. This study was approved by the ethics committee of the Faculty of Medicine, Universitas Indonesia, and informed consent was obtained from all patients.

Each paraffin block was cut into 4 μm thick sections on a microtome, after which, the sections were placed on poly-l-lysine-coated glass slides, which were then heated on a hot plate at 55–58 °C. The samples were deparaffinized in a graded series of xylol and rehydrated in a graded series of alcohol. The slides were subjected to heat-induced antigen retrieval using 0.1 M NaOH citrate buffer (pH 7.0) in an autoclave at 121 °C for 15 minutes and washed with phosphate-buffered saline (PBS; pH 7.4) for five minutes. Endogenous peroxidase was blocked with hydrogen peroxide in methanol (3% v/v) for 30 minutes at room temperature. The slides were then washed under running tap water for five minutes, and nonspecific proteins were blocked with Background Sniper (Biocare Medical) for 15 minutes.

The slides were incubated overnight with primary antibodies to OCT4 (Santa Cruz Biotechnology; dilution 1:150), SOX2 (Santa Cruz Biotechnology; 1:300), or hTERT (Abcam; 1:3000). Some slides were incubated with antibodies to caspase-3 (Abcam; 1:200) and Chk1 (Abcam; 1:1000) for one hour. After the appropriate incubation, the slides were washed with PBS for five minutes, and for SOX2, OCT4, and hTERT staining, the slides were left overnight and then washed with PBS for five minutes.

Each slide was incubated with the appropriate biotinylated secondary antibody (Trekkie Universal Link) for 15 minutes. Diaminobenzene was added, and the slides were incubated for two minutes. The slides were then counterstained with Mayer's hematoxylin (Lillie's modified; Roche) for two minutes. Positive and negative controls were included for each antibody.

The immunohistochemistry slides were scanned entirely under low-power magnification (40 ×) on a Leica ICC 50 HD microscope, and 10 representative areas were photographed under high-power magnification (400 ×). The images were evaluated manually using Image J (cell counter) by two pathologists. The expression of SOX2, OCT4, Chk1, caspase-3, and hTERT was evaluated for each patient using an H-score as a semiquantitative approach by determining the staining intensity as strong (3+), moderate (2+), weak (1+) positive, or negative. One thousand cells were examined for each slide to provide a representative score for each sample. The H-score was calculated as (1 × (% cells

Table 1: Patients' characteristics and therapy responses.

Variables	Initial therapy response, n (%)[a]		Final therapy response, n (%)[b]	
	Partial, n = 15	Good, n = 25	Incomplete, n = 21	Complete, n = 19
Age, years				
< 45	2 (13.3)	3 (12.0)	3 (14.3)	2 (10.5)
45–63	11 (73.3)	18 (72.0)	16 (76.2)	13 (68.4)
> 63	2 (13.3)	4 (16.0)	2 (9.5)	4 (21.1)
Differentiation				
Well	1 (6.7)	5 (20.0)	3 (14.3)	3 (15.8)
Moderate	9 (60.0)	17 (68.0)	12 (57.1)	14 (73.7)
Poor	5 (33.3)	3 (12.0)	6 (28.6)	2 (10.7)
Therapy				
Radiation	12 (80.0)	24 (96.0)	18 (85.7)	18 (94.7)
Chemoradiation	3 (20.0)	1 (4.0)	3 (14.3)	1 (5.3)

[a]Response after finishing radiation/chemoradiation. [b]Response 6–12 months after therapy.

1+) + 2 × (% cells 2+) + 3 × (% cells 3+)), which gave a result with a range of 0–300. The mean score for each antibody was used as a cutoff to identify weak or strong expression for each slide.

The patients' characteristics, radiotherapy response, immunohistochemistry results, and follow-up data were analyzed. The data were analyzed using univariate and inferential statistics and presented as frequencies and percentages for each variable. The data were also analyzed for comparisons using bivariate analysis such as the chi-square test. Multivariate analysis included logistic regression analysis to identify those factors associated with the final therapy response. For all data, a p-value ≤ 0.050 was considered to indicate significance.

RESULTS

This study involved 40 samples from 19 control subjects and 21 patients. Two pathologists performed immunohistochemistry evaluation; their kappa score was 0.750, which indicated good agreement between the two pathologists. The most frequent age group was 45–63 years (72.5%). All samples were stage IIIB cervical cancer with the same type of histopathology (i.e., squamous cell carcinoma (SCC)). All samples were nonkeratinizing SCC because the few keratinized samples were excluded; most (65.0%) were moderately differentiated. All patients received radiation, and four (10.0%) patients received combined radiation and chemotherapy [Table 1].

More than half of the samples (73.3%) from patients with a partial initial treatment response showed strong expression of SOX2 (H-score > 96.6). Similarly, 86.7% of samples from patients with a partial initial treatment response showed strong expression of OCT4 (H-score > 61.9).

A high percentage of samples from patients with an incomplete response to the final treatment

Table 2: Expression of SOX2 and OCT4, and initial and final therapy responses.

CSC marker expression	Initial therapy response				Final therapy response			
	Partial, n (%)	Complete, n (%)	p-value	OR (95% CI)	Incomplete, n (%)	Complete, n (%)	p-value	OR (95% CI)
SOX2								
High	11 (73.3)	8 (32.0)	0.011[a]	5.80 (1.41–24.17)	15 (71.4)	4 (21.1)	0.001[a]	9.40 (2.19–40.11)
Low	4 (26.7)	17 (68.0)			6 (28.6)	15 (78.9)		
OCT4								
High	13 (86.7)	8 (32.0)	0.001[a]	13.80 (2.50–76.33)	17 (81.0)	4 (21.1)	< 0.001[a]	15.90 (3.38–75.10)
Low	2 (13.3)	17 (68.0)			4 (19.0)	15 (78.9)		

[a]chi-square test. CSC: cancer stem cell; OR: odds ratio; CI: confidence interval; SOX2: sry-related HMG box; OCT4: octamer binding transcription factor 4.
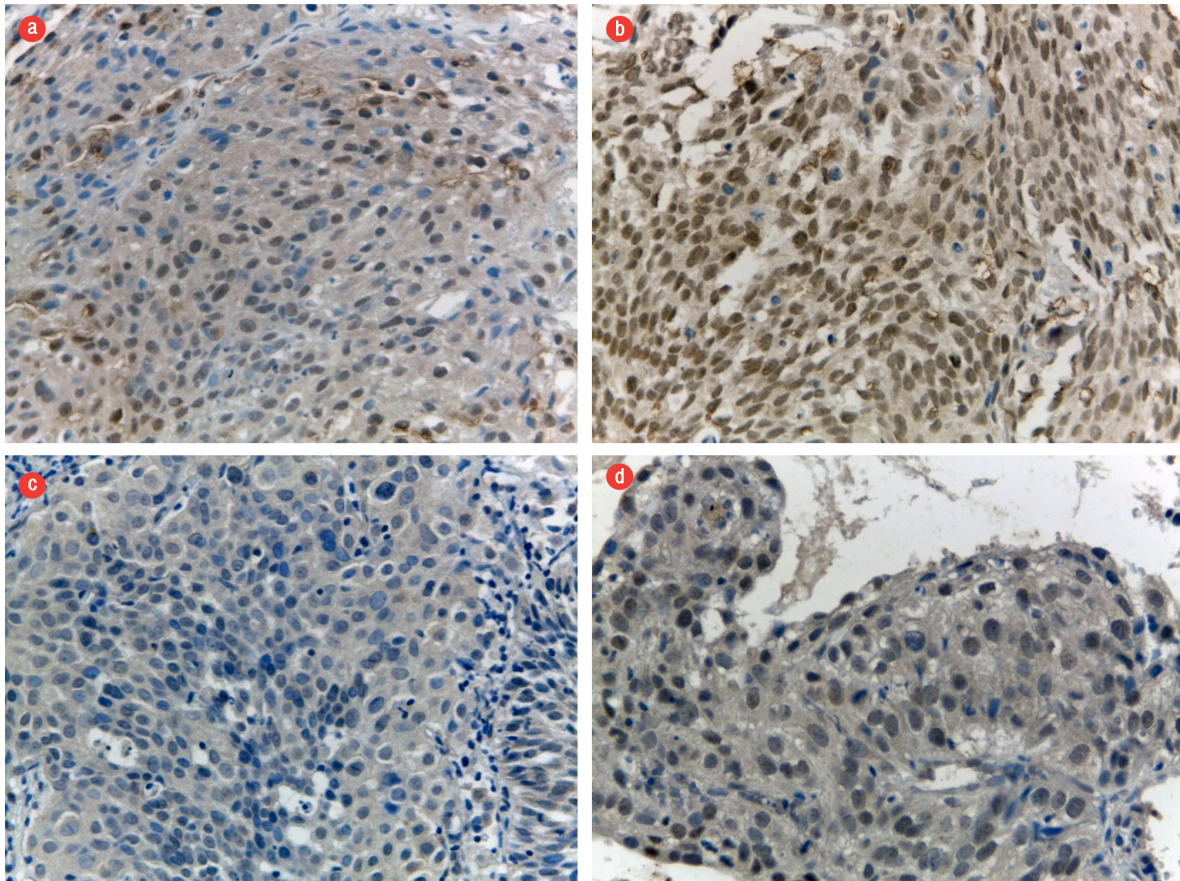
Figure 1: Expression of SOX2 and OCT4. Representative examples with **(a)** low and **(b)** high nuclear expression of SOX2, and **(c)** low and **(d)** high nuclear expression of OCT4. Magnification = 400 ×.

exhibited strong expression of SOX2 and OCT4 (71.4% and 81.0%, respectively).

Table 2 shows the expression of SOX2 and OCT4 differed significantly between patients with a partial initial or complete treatment response: SOX2 odds ratio (OR) = 5.80, 95% confidence interval (CI): 1.41–24.17; p = 0.011) and OCT4 (OR = 13.80, 95% CI: 2.50–76.33; p = 0.001). The expression of SOX2 and OCT4 also differed significantly between patients with a partial or complete final treatment response: SOX2 (OR = 9.40, 95% CI: 2.19–40.11; p = 0.001) and OCT4 (OR = 15.90, 95% CI: 3.38–75.10; p < 0.001). Figure 1 shows the immunohistochemical staining of SOX2 and OCT4

Table 3: Relationships between SOX2, OCT4, Chk1, caspase-3, and hTERT expression.

Other markers	SOX2			OCT4		
	Low, n (%)	Strong, n (%)	p-value	Low, n (%)	Strong, n (%)	p-value
Chk1						
Low	17 (81.0)	13 (68.4)	0.473[b]	15 (78.9)	15 (71.4)	0.721[b]
Strong	4 (19.0)	6 (31.6)		4 (21.1)	6 (28.6)	
Caspase-3						
Positive	3 (14.3)	5 (26.3)	0.442[b]	3 (15.8)	5 (23.8)	0.698[b]
Negative	18 (85.7)	14 (73.7)		16 (84.2)	16 (76.2)	
hTERT						
Low	11 (52.4)	9 (47.4)	0.752[a]	8 (42.1)	12 (57.1)	0.342[a]
Strong	10 (47.6)	10 (52.6)		11 (57.9)	9 (42.9)	

[a]chi-square test. [b]Fisher's exact test. SOX2: sry-related HMG box; OCT4: octamer binding transcription factor 4; Chk1: checkpoint kinase 1; hTERT: human telomerase reverse transcription.
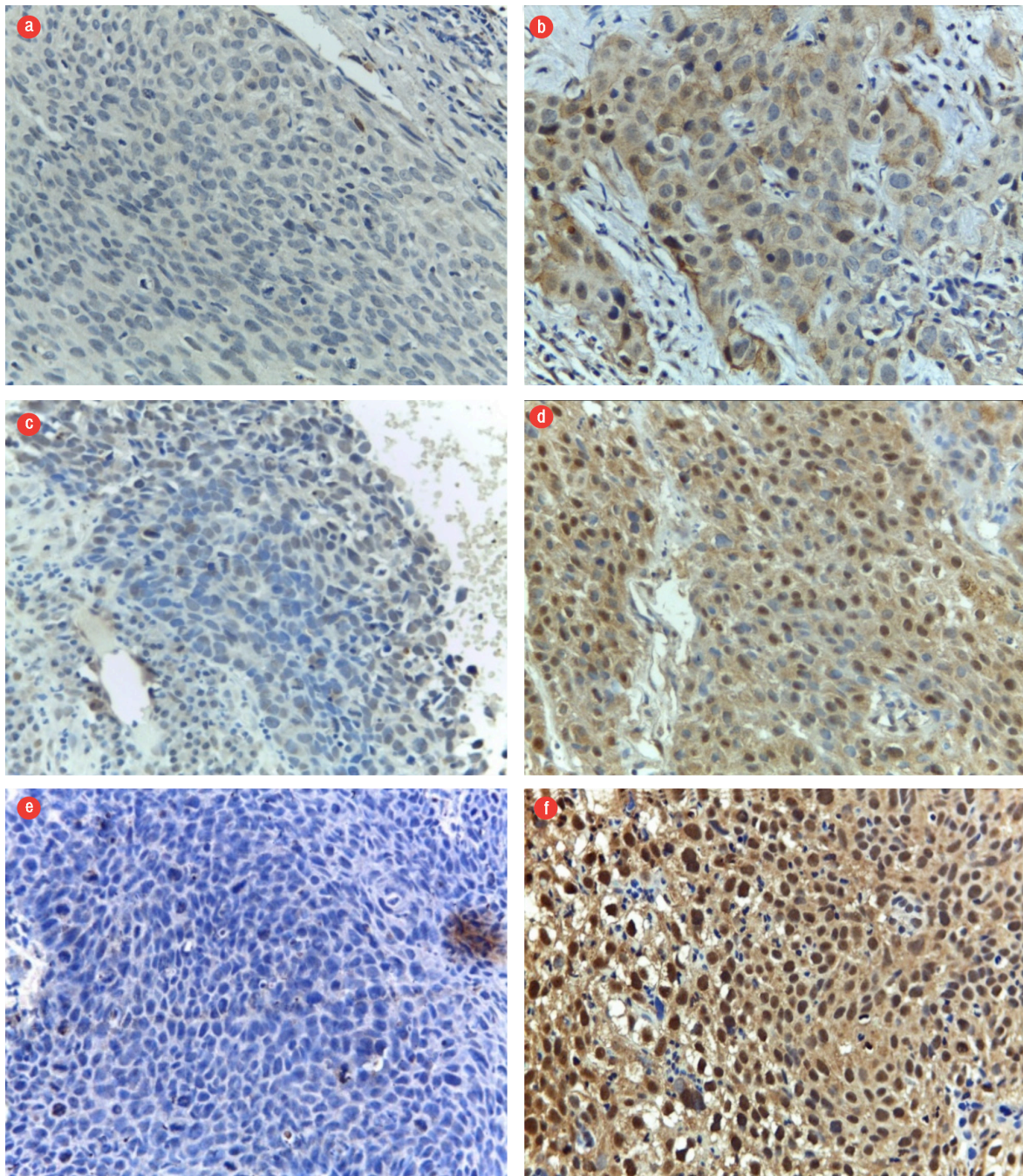
Figure 2: Expression of caspase-3, Chk1, and hTERT. **(a)** Negative and **(b)** positive expression of caspase-3 in the cytoplasm. **(c)** Low and **(d)** high expression of Chk1 in the nucleus. **(e)** Low and **(f)** high expression of hTERT in the nucleus. Magnification = 400 ×.

expression. Figure 2 shows the patterns of Chk1, caspase-3, and hTERT expression.

The expression levels of Chk1, caspase-3, and hTERT as markers of CSCs were compared with those of SOX2 and OCT4 [Table 3]. The expression patterns of these markers did not differ significantly from those of SOX2 and OCT4 ($p > 0.050$).

Logistic regression multivariate analysis was used to identify the association between markers and the final treatment response. The levels of SOX2 (OR = 5.12, 95% CI: 1.08–24.39; $p = 0.034$) and OCT4 (OR = 17.03, 95% CI: 3.58–81.15; $p = 0.004$) expression were significantly associated with the six to 12-month post-radiation response in patients with stage IIIB cervical SCC [Table 4].

The probabilities of exhibiting an incomplete response to the final therapy according to the expression levels of SOX2 and OCT4 were as

Table 4: Logistic regression to identify markers of the final therapy response.

Markers	Bivariate analysis		Multivariate analysis	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
SOX2	9.38 (2.19–40.11)	0.001	5.12 (1.08–24.39)	0.034
OCT4	15.94 (3.38–75.10)	< 0.001	17.03 (3.58–81.15)	0.004
Chk1	2.67 (0.58–12.33)	0.281	-	-
Caspase-3	0.60 (0.12–2.94)	0.698	-	-
hTERT	0.55 (0.16–1.91)	0.342	-	-

OR: odds ratio; CI: confidence interval; SOX2: Sry-related HMG box; OCT4: octamer binding transcription factor 4; Chk1: checkpoint kinase 1; hTERT: human telomerase reverse transcription.

follows: strong expression of both SOX2 and OCT4, 87.72%; strong expression of OCT4 and weak expression of SOX2, 54.02%; strong expression of SOX2 and weak expression of OCT4, 38.58%; and weak expression of both SOX2 and OCT4, 9.28%.

Six patients (24.0%) had a good initial response to therapy, but this changed to incomplete therapy after 6–12 months because of inguinal and collie lymph nodes metastases, abdominal wall metastases, pleural effusion, and ascites, as confirmed in cytological or histopathological specimens. One patient (4.0%) was diagnosed with SCC based on the Pap smear result.

DISCUSSION

Cervical cancer is a major health problem in Indonesia. This study included 40 patients with stage IIIB cervical cancer; most patients were aged 45–63 years (72.5%). Histopathologically, all samples were diagnosed as nonkeratinizing SCC. Similar findings were reported in a study from Korea, which reported the most common age group as 19–83 years old and SCC as the most common cancer.[12] Previous studies reported that SCC was more frequent in women older than 50 years.[13–15] The 45–63 years age range found in our study fits the age range reported by others.[13,14]

High expression levels of SOX2 and OCT4 are associated with radioresistance of SCC.[16] Kumazawa et al,[17] also reported morphological differences between the side population (another term for CSC) and non-side population after patients received radiation at a dose of up to 6 Gy. The non-side population colony became separated after radiation treatment. Cells exhibiting positive expression of SOX2 had a greater capacity for self-renewal, differentiation, and tumor formation.[18] Some molecular mechanisms may explain the association between CSC existence and radioresistance. The

basic principle of radiotherapy has been described as the four "Rs" of radiobiology: repair, redistribution, repopulation, and reoxygenation.[19]

The CSC response to radio fraction is different from that of non-CSCs. The tumor volume of non-CSC tumors decreases because of DNA damage induced by ionizing radiation. In contrast, CSCs show more active repair of damaged DNA after radiation. The ability of self-renewal through ATM and Chk1/2 is more efficient in CSCs.[20] Fast repopulation during and after radiotherapy is an important reason for failed radiotherapy.[21,22] CSCs exhibit increased activities of glutamate cysteine ligase and glutathione synthetase. These changes seem to facilitate an increase in the availability of scavengers (i.e., the content of reactive oxygen species and double-strand breaks of DNA is lower in CSCs than in non-CSC populations in response to the same dosage of radiation).[20]

Apoptosis (via caspase-3), DNA repair (Chk1), and telomerase (hTERT) play roles in carcinogenesis, including that involving SCC. We found no significant differences between expression levels of caspase-3, Chk1, and hTERT, or between those of SOX2 and OCT4, which suggests that the differences in response to therapy might be affected by other molecular factors.[23] Clinically, two markers of CSCs—SOX2 and OCT4—should be tested together because each had a different probability of incomplete final therapy response. The probability of DNA repair (Chk1) should also be considered when determining the treatment plan for uterine cervical cancer (e.g., the combination of chemoradiation and an inhibitor of Chk1).[24]

CONCLUSION

In patients with keratinizing SCC, expression of SOX2 and OCT4 was significantly associated with a

partial initial response to therapy. A high expression level of SOX2 or OCT4 was significantly associated with a partial initial therapy response; the probability of an incomplete final response to therapy was 87.9% in patients with a strong expression of both markers. The patterns of caspase-3, Chk1, and hTERT expression did not differ from those of SOX2 and OCT4. High expression levels of SOX2 and OCT4 suggest an incomplete radiotherapy outcome in patients with stage IIIB cervical cancer.

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