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Original Article

Application of histone modification in the risk prediction of the biochemical recurrence after radical prostatectomy

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Abstract

The role of histone modifications in the development and progression of cancer remains unclear. Here, we gave an investigation of the relationship between the various histone modifications and the risk prediction of the biochemical recurrence after radical prostatectomy (RP). Histone 3 lysine 4 dimethylation (H3K4diMe), trimethylation (H3K4triMe), lysine 36 trimethylation (H3K36triMe), histone 4 lysine 20 trimethylation (H4K20triMe) and acetylation of histome 3 lysine 9 (H3K9Ac) were evaluated using immnuohistochemistry coupled with the tissue microarray technique in 169 primary prostatectomy tissue samples. Recursive partitioning analysis (RPA) was used to analyze the data. Through global histone modification analysis in patients who underwent radical prostatectomy, we found that H3K4triMe can predict the risk of the biochemical recurrence for the low grade prostate cancer (Gleason score $\leq$ 6) after RP. In the case of high grade prostate cancer (Gleason score $\geq$ 7), H4K20triMe and H3K9Ac accompanying with the pre-operation prostate-specific antigen (PSA) level could also predict the risk of the biochemical recurrence after RP. In combination with the Gleason score and pre-operation PSA level, the acetylation and methylation of histones H3 and H4 can predict the biochemical recurrence of the prostate cancer following RP.

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Keywords: biochemical recurrence, histone modification, immunohistochemistry, prostate cancer, radical prostatectomy, tissue microarray.

1 Introduction

The advent of prostate-specific antigen (PSA) testing in the late 1980s has resulted in the increasing diagnosis and treatment of lower stage prostate cancer, both in the US and in Europe [1], and improved the proportion of men receiving the radical prostatectomy (RP) for organ-confined diseases significantly [2]. Due to the combination of increasingly refined surgical techniques, reduced incidence of surgical complications and decreased mortality, many patients with localized prostate cancer have experienced long-term survival [3]. However, the biochemical recurrence, which is generally the earliest indicator of recurrent disease, occurs in about 25%–50% of patients following RP on long-term follow-up [4]. Therefore, the early risk detection of the biochemical recurrence may improve the prognosis of these patients.

Currently, the prediction of the clinical outcome of prostate cancer patients who have undergone RP is

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based mainly on the pre-operation PSA level, Gleason score, and the pathological stage [5]. However, prostate cancers with equivalent PSA level, Gleason score, and pathological stage may have different clinical outcomes, for the molecularly heterogeneous subtypes [6]. Therefore, it is crucial to find out more effective biomarkers to determine the prognosis of the localized prostate cancer after RP.

In addition to the genetic alterations, epigenetic changes may also contribute to the development and progression of cancer and leukemia [7]. The epigenetic deregulation indicates the alterations in the methylation status of DNA, the covalent modification of histone tails, the chromatin remodeling and microRNAs [8, 9]. It is likely to be linked to the patient prognosis due to the influence on several aspects of tumor cell biology, such as the cell growth, differentiation and cell death [10, 11]. Among the components of the epigenetic changes, the histone modification is relatively less characterized. Histone-modifying enzymes will affect histones either locally through the targeted recruitment by sequence specific transcription factors [6, 12], or globally via the genome in an untargeted manner affecting virtually all nucleosomes [13]. Like their targeted effects, the global activity of histone modifying enzymes can also modulate the gene activity [14]. Therefore, the local and global histone modifications will generate the hierarchical patterns of the modifications from single promoters to large regions of chromosomes and even single cell [15], through the multiple histone-modifying enzymes with different substrate specificities. The histone modifications will differ depending on the region of the chromatin, the cell type, the tissue type, and the external conditions of a cell, thus the prostate cancers would have typical patterns of histone modifications [16]. Since the deregulation of histone modifications at the level of a single promoter is intimately related to the mis-expression of the downstream gene, which in most cases cannot provide adequate information to predict clinical outcome[6, 17], none of the locus-specific changes in histone modifications has so far been related usually or causally to the clinical outcome [18, 19]. The recent work by Seligson *et al*. [20] suggested that some various histone modification patterns can predict the clinical outcome of the low-grade (Gleason score ≤ 6) prostate cancer following RP, and the information on global patterns of other modification sites will probably help with the further classification of all patients due to the substantial number of modifications on histone, even for those in the high-grade category. In this investigation, our data suggested that the risk of the biochemical recurrence of the localized prostate cancer after RP, including both low- and high-grade patients, was associated with the global patterns of histone modifications.

2 Materials and methods

2.1 Patients

Tumor samples from 169 prostate cancer patients were included in the study. These patients underwent RP and regional lymph node dissection were from Renji Hospital, subsidiary of Shanghai Jiao Tong University School of Medicine, between January 2001 and December 2008. They did not receive any pre-operation treatment. The histopathologic features of tumor specimens were classified according to the World Health Organization (WHO) criteria [21, 22].

A retrospective analysis for the outcome assessment was based on the detailed anonymized clinico-pathological information linked to the tissue microarray (TMA) technique specimens.

2.2 Immunohistochemical analysis and prostate TMA

We used the specific rabbit polyclonal antibodies to detect five different histone modifications, which included H4K20triMe, H3K36triMe (Abcam, Cambridge, UK), H3K4diMe, H3K4triMe, and H3K9Ac (Cell Signaling Technology, Danvers, MA, USA). The antibodies were tested and optimized on the whole-tissue sections and test arrays. Once an appropriate dilution and incubation time was determined, two tissue array sections containing all patient samples were stained for each antibody, through the standard two-step immunohistochemistry.

Tissue array sections were cut with a sectioning aid (Instrumedics, St. Louis, MO, USA) immediately before being stained, and then they were rehydrated in the graded alcohols after the deparaffinization in xylenes. The endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol at room temperature (24ºC), and the sections were placed in a 120ºC, 0.01 mol L^{-1} sodium citrate buffer (pH 6.0) for antigen retrieval. The primary antibodies were applied at the following dilutions: H4K20triMe at 1:300, H3K36triMe at 1:1 500, H3K4diMe at 1:750, H3K4triMe at 1:50, and H3K9Ac at 1:200. Dako Envision System (Dako, Carpinteria, CA, USA) was then used as the second antibody. The sections were visualized with diaminobenzidine (DAB), counterstained with

hematoxylin, dehydrated, and mounted. The identical array sections stained in the absence of the primary antibody served as the negative controls.

A semi-quantitative assessment of the antibody staining on the TMAs was performed independently by two observers, who were blinded to all the clinicopathologic variables. The frequency of nuclear positive target cells (range 0%–100%) in prostatic glandular epithelium was scored for each TMA spot.

The prostate TMA was constructed with formalin-fixed, paraffin-embedded prostate tissue samples, and the areas of the invasive adenocarcinoma were identified according to the corresponding hematoxylin and eosin (H&E) stained slides. Two replicate tumor samples (1 mm in diameter) were taken from the donor tissue blocks in a highly representative fashion and arrayed into a recipient paraffin block (35 mm × 622 mm × 65 mm) using a tissue microarrayer (Gentury, Beecher, Maryland, USA), as described by Kononen *et al.* [23].

2.3 Statistical analysis

Follow-up data were updated in December 2008, and the biochemical recurrence-free survival time was defined as the time from the date of surgery to the date of the biochemical recurrence. By using the Kaplan-Meier method, the probability of survival could be estimated. At the same time, we can analyze the differences in the survivals through the log-rank test. To determine whether the variables differ across the groups, the χ^2 test or Fisher's exact test was used according to the test condition. A Cox proportional hazards model was performed to establish independent factor(s) for survival. Statistical significance was defined as $P < 0.05$, and all of the tests were two-sided. The statistical analysis was performed based on the SPSS software package, version 15.0 (SPSS Inc., Chicago, IL, USA).

To explore how the unique patterns of the histone modification would influence the prognosis of the localized prostate cancer patients after RP, we applied the recursive partitioning analysis (RPA) to the data [24, 25], which was performed through the rpart package of the freely available software [26].

3 Results

3.1 Patient characteristics

One hundred and sixty-eight patients were totally available for the analysis, among which some patients missed clinical data and one patient was not included due to the lacking of follow-up data. The typical clinical characteristics were summarized and presented in Table 1. Biochemical recurrence was defined as a post-operative serum PSA concentration ≥ 0.2 ng mL^{-1} [27], which was seen in 99/168 (58.9%) of all the studied patients, in 67/94 (71.3%) of the patients with high-grade tumors, and in 32/74 (43.2%) of the patients with low-grade tumors, it was obvious that the high-grade tumors group was more likely to have biochemical recurrence ($P < 0.001$). The follow-up was defined as the time from the date of the surgery to the date of the biochemical recurrence or to the last contact in non-recurring patients. In the survival analysis, median biochemical recurrence-free time was lower in the high-grade tumors group than that in the low-grade one (24.5 months *vs*. 35 months, log-rank $P < 0.001$). For the patients with low-grade tumors, the follow-up within the recurring and non-recurring patient groups was 30 months (range 2–53 months) and 48.5 months (range 22–76 months), respectively. However, for those with high-grade tumors, the follow-up was 21 months (range 2–63 months) and 32 months (range 18–68 months) in the recurring and non-recurring patient groups, respectively.

Through the χ^2 analysis or Fisher's exact test, we found that only some parameters had statistical differences between the high-grade tumor group and low-grade tumor group, such as the pathological stage (pT), lymph node status, tumor margins, organ confined, pre-operation PSA, bio-Gleason score, recurrence, total follow-up, and total follow-up in non-recurring group ($P < 0.05$).

3.2 Acetylation and methylation of H3 and H4 in prostate cancer tissue samples

Figure 1 (A–E) showed the representative staining of the H4K20triMe, H3K4diMe, H3K4triMe, H3K36triMe, and H3K9Ac on tissue arrays, and the frequency of positive cells stained by the anti-H4K20triMe, -H3K4diMe, -H3K4triMe, -H3K36triMe, and -H3K9Ac was further determined (Figure 1F). We found that the overall median expression of H4K20triMe, H3K4diMe, H3K4triMe, H3K36triMe, and H3K9Ac in the localized prostate cancer tissue samples was 70%, 58%, 35%, 30%, and 65%, respectively. Based on the statistical analysis, the Gleason score corresponding to the levels of five histone modifications for all these tissue samples were indicated as the follows: H3K4diMe ($r = -0.086$, $P = 0.265$), H3K4triMe ($r = 0.064$, $P = 0.408$),

H3K36triMe (r = –0.056, P = 0.470), H4K20triMe (r = –0.084, P = 0.279), and H3K9Ac (r = –0.146, P = 0.058). Therefore there were no significant relationships between any of the histone modifications and the Gleason score.

Table 1. Clinicopathological parameters in low- and high-grade prostate cancer.

	All low grade (n = 74)	All high grade (n = 94)
[a]Age at surgery (n = 168)		
Median (range)	68.0 (52.0–77.0)	67.0 (48.0–77.0)
Mean	67.31	65.56
[b]Pathology pT staged (n = 168)*		
PT2-pT3a	71	79
PT3b	3	15
[c]Lymph node status (n = 168)*		
Positive	1	8
Negative	73	86
[b]Tumor margins (n = 168)*		
Positive	4	20
Negative	70	74
[c]Capsular invasion (n = 168)		
No invasion	73	90
Invasion	1	4
[b]Organ confined (n = 168)*		
Yes	3	21
No	70	73
[a,d]PreOpPSA (ng mL^{-1}) (n = 168)*		
Median (range)	14.00 (1.43, 59.12)	21.00 (2.61, 100)
Mean	17.8543	25.1349
[a,d]Bio-gleason score*		
Median (range)	6.0 (3.0, 9.0)	8.0 (3.0, 9.0)
Mean	6.432	7.2316
[b]Recurrence (n = 168)*		
Yes (≥ 0.2 ng mL^{-1})	32	67
No (< 0.2 ng mL^{-1})	42	27
[a,d]Total follow-up (months)*		
Median (range)	35.00 (2.00, 76.00)	24.50 (2.00, 68.00)
Mean	39.01	25.85
[a,d]Total follow-up in recurred group (n = 99)		
Median (range)	30.00 (2.00, 53.00)	21.00 (2.00, 63.00)
Mean	30.03	22.23
[a,d]Total follow-up in non-recurring group (n = 69)*		
Median (range)	48.50 (22.00, 76.00)	32.00 (18.00, 68.00)
Mean	45.86	35.84

*P < 0.05, compare the clinicopathological parameters between the low grade and high grade patients. a: Mean (t-test), b: χ^2 test, c: Fisher's exact test, d: Mann-Whitney U test.

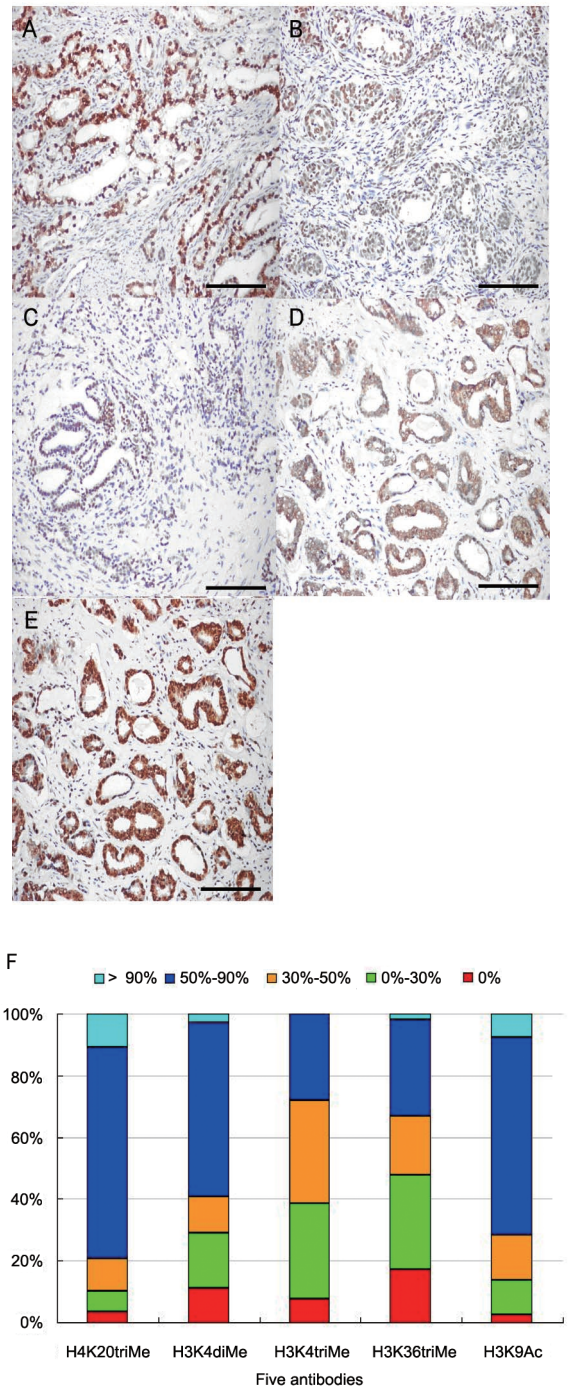

Figure 1. Immunohistochemical analysis of histone modification in prostate tumors. Representative images of H4K20triMe (A), H3K4diMe (B), H3K4triMe (C), H3K36triMe (D), and H3K9Ac (E) staining of the prostate tumors on tissue arrays. Scale bars = 500 μm. The cutline (F) represents the distribution of positive cell (%) for the five different antibodies across all 168 tissue samples, the y-axis is the fraction of samples showing positive cell for the indicated percentage of cells.

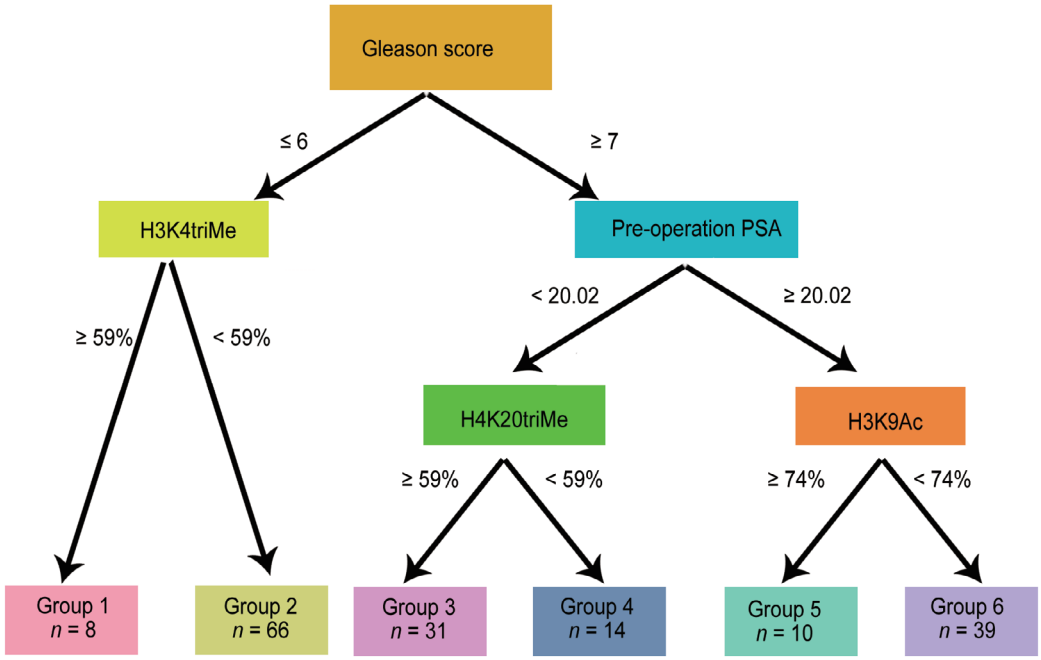

Figure 2. Six distinct prognostic groups classified by recursive partitioning analysis (RPA), according to the Gleason score, pre-operation prostate-specific antigen (PSA) levels, and histone modification.

Table 2. Median biochemical recurrence-free time and five-year biochemical recurrence-free survival rates post-radical prostatectomy (RP) for each group of prostate cancer patients established by recursive partitioning analysis (RPA).

Group	No. of patients	Biochemical recurrence-free		Five-year biochemical recurrence-free survival rates (%)
		Median (months)	95% CI	
1	8	*	*	87.5
2	66	49.00	38.95 – 59.05	40.8
3	31	42.00	36.78 – 47.22	43.5
4	14	29.00	16.17 – 41.83	14.3
5	10	27.00	19.01 – 34.99	0.00
6	39	19.00	14.11 – 23.89	0.00

$P = 0.000$, using the Kaplan-Meier method to estimate the probability of survival and comparing the median biochemical recurrence-free time and five-year biochemical recurrence-free survival rates among the six groups of the patients after RP established by RPA through the log-rank test.
*Because the recurrence rate of the group 1 has not dropped below 50 percent, the median time and 95% CI cannot be estimated.

3.3 Global histone modification patterns and the biochemical recurrence of prostate cancer

RPA was used to explore whether global histone modifications were involved in between subsets of the tissue samples. As shown in Figure 2, six distinct prognostic groups were classified by RPA, according to the Gleason score, pre-operation PSA levels, and histone modifications. The median biochemical recurrence-free time and five-year biochemical recurrence-free survival rates post-RP for each group of prostate cancer patients were shown in Table 2. Based on Gleason score provided for the first prognostic node, we found that the prostate cancer patients with Gleason score ≥ 7 (high-grade) had higher risk of biochemical recurrence than those with Gleason score ≤ 6 (low-grade) (Figure 2).

The patients with Gleason score ≤ 6 (low-grade) were then classified into two separate groups by a second node of H3K4triMe. We could also found that the patients with more than or equal to 59% of

tumor cells expressing H3K4triMe was susceptible to experience biochemical recurrence risk compared with the patients whose less than 59% tumor cells expressing H3K4triMe (Figure 2; group 2 *vs*. group 1).

For the patients with Gleason score ≥ 7 (high-grade), RPA could also provide a second node based on PSA levels. these patients with pre-operation PSA concentrations ≥ 20.02 had a worse prognosis than those with pre-operation PSA < 20.02. Interestingly, RPA incorporated histone modifications could be characterized as a third node for these patients. H4K20triMe significantly influenced the survival of high-grade patients with pre-operation PSA < 20.02, with a greater survival for the patients whose tumors expressing higher levels (≥ 59% of tumor cells) of H4K20triMe (Figure 2; group 3 *vs*. group 4). Moreover, H3K9Ac largely affected the survival of high-grade patients with pre-operation PSA ≥ 20.02, with a greater survival for the patients whose tumors expressing higher levels (≥ 74% of tumor cells) of H3K9Ac (Figure 2; group 5 *vs*. group 6).

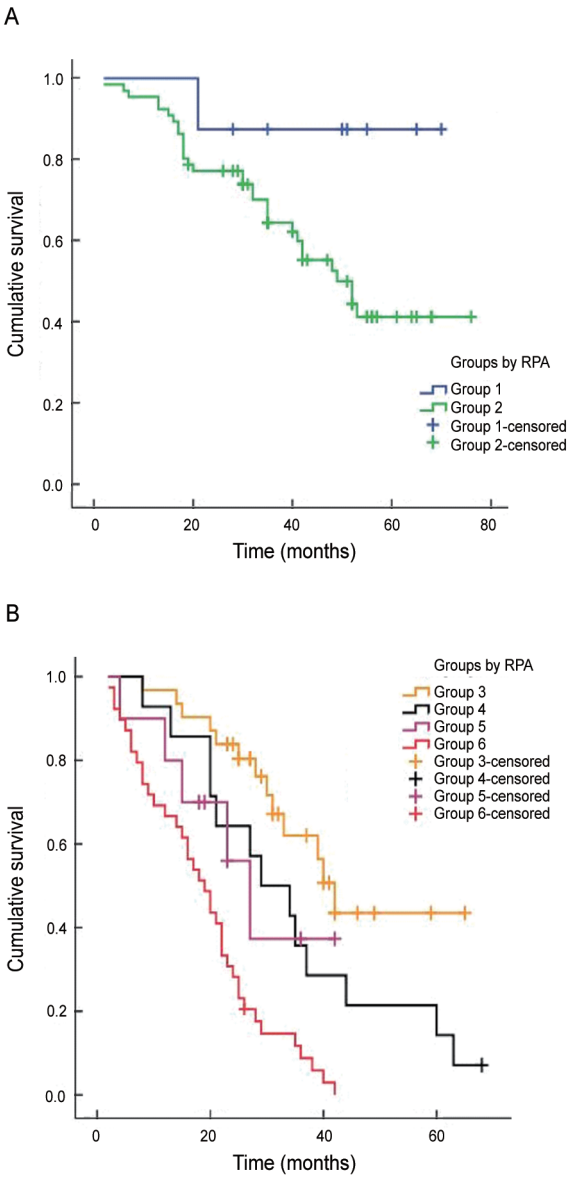

Figure 3. Biochemical recurrence-free survival for each of the six groups defined by recursive partitioning analysis (RPA) analysis.

To confirm whether the identified groups were clinically significant, we determined the risk of biochemical recurrence in each group after the removal of the primary tumor through the Kaplan-Meier method (Figure 3). The five-year biochemical recurrence-free survival rates of patients with the low-grade tumor group 1 and group 2 were 87.5% and 40.8% (P = 0.000), respectively (Figure 3A), while those patients with the high-grade tumor group 3, group 4, group 5, and group 6 were 43.5%, 14.3%, 0.0% and 0.0% (P = 0.000), respectively (Figure 3B). We employed a Cox proportional hazards model to assess the independent predictors for the biochemical recurrence-free survival, which included age and RPA classification. Gleason score, pre-operation PSA, tumor margins, and pathologic stage were not retained by the model due to the co-linearity with RPA group variable. COX regression analysis showed that: the RPA group 1 for the reference group, the groups 4, 5, 6 adjusted after the age factor had a higher hazard ratio, and there were statistical significance (Table 3).

Table 3. Multivariate analysis using a Cox proportional hazards model including the groups determined by the RPA and age.

Group	Hazard Ratio	95% CI	P value
1	Reference		
2	4.977	0.677 – 36.579	0.115
3	5.711	0.743 – 43.897	0.094
4	11.362	1.485 – 86.953	0.019
5	12.661	1.464 – 109.527	0.021
6	31.732	4.295 – 234.453	0.001
Age ≥ 70	0.787	0.245 – 2.532	0.688

Note: Gleason score, pre-operation PSA, tumor margins, risk and pathologic stage were not retained by the model due to co-linearity with the recursive partitioning analysis (RPA) group variable. Multivariate analysis showed that the risk of groups 4, 5, 6 is higher than the control group (group 1).

4 Discussion

In addition to the changes in the DNA sequence, the epigenetic alterations (DNA methylation, histone modification, and so on) also play important roles in the carcinogenesis by controlling the gene activity and nuclear architecture [28]. During the prostate carcinogenesis, epigenetic changes are so frequent that they allow the discrimination between the normal and neoplastic tissues with a specificity of up to 100% [29, 30], some histone modifications are associated with the increased risk of low-grade prostate cancer recurrence [20]. However, the relationship between global histone modification and the prostate cancer progression remains elusive [31].

In this study, we used the immunohistochemical analysis to evaluate the pattern of the histone modifications in prostate cancer and its correlation with the prognosis of the prostate cancer biochemical recurrence after RP. We found that H3K4triMe influenced the risk of the biochemical recurrence of the low-grade (Gleason score ≤ 6) prostate cancer patients who had undergone RP. The patients whose tumors expressed H3K4triMe less than 59% of tumor cells had higher risk of biochemical recurrence than those with tumors more than or equal to 59% of cells expressing H3K4triMe (Figure 2; group 2 *vs*. group 1, Table 2). Specifically, through the RPA analysis, five-year biochemical recurrence-free survival rates of group 1 and group 2 were 87.5% and 40.8%, respectively. These results were similar to those previously reported by Seligson *et al.* [20]. Furthermore, for the high-grade prostate cancer, our study revealed that in combination with the pre-operation PSA levels, H4K20triMe and H3K9Ac could predicate the biochemical recurrence of prostate cancer patients with Gleason score ≥ 7 (high-grade). Among the high-grade patients with PSA levels < 20.02, the patients whose tumors expressing higher levels ($\geq$ 59% of tumor cells) of H4K20triMe experienced greater survival than the patients whose tumors expressing lower levels (< 59% of tumor cells) of H4K20triMe (Figure 2; group 3 *vs*. group 4). While for those high-grade patients with pre-operation PSA ≥ 20.02, the H3K9Ac significantly influenced the survival. Compared with the patients whose tumors expressing lower levels (< 74% of tumor cells) of H3K9Ac, those expressing higher levels ($\geq$ 74% of tumor cells) of H3K9Ac (Figure 2 group 5 *vs*. group 6) had higher survival rate. On the basis of our results, the histone modifications, especially for the methylation and acetylation of histone H3 and H4, should be considered in the attempt to better define the prognostic subgroups in the prostate cancer patients.

The biochemical recurrence rate after radical prostatectomy is relatively high in our study. The recurrences of the patients after radical prostatectomy in many literatures were quite different, because of the number of cases, follow-up period, surgical techniques and diagnostic criteria of biochemical recurrence were different. Sakr *et al*. [32] studied 534 patients after radical operation and found the biochemical recurrence of the patients was 40%. Recently, Ricethe et al. have reported that biochemical recurrence rate is 24.2%–38.5% among 1 159 patients after radical prostatectomy [33]. Generally, biochemical recurrence rates reported in the literature are between 30–50 percent [4, 34]. Some studies showed that the biochemical recurrence after radical prostatectomy was related closely to the surgical techniques [35]. Radical prostatectomy has been launched in china since 1990, while surgical technique is still not perfect. In our study the high biochemical recurrence rate after radical prostatectomy is related to the immature surgical skills.

Gleason score was selected as the first node, due to its well-known prognostic in the prostate cancer. Therefore, the histone modifications retained by the RPA should be regarded as a valuable, new prognostic factor in prostate cancer patients, especially for the histone H3K4triMe related to the prognosis of those low-grade prostate cancer patients who had undergone RP, as well as H4K20triMe and H3K9Ac related to the prognosis of the high-grade prostate cancer patients who had undergone RP. However, how these specific epigenetic modifications or a combination of modifications affect the expression of particular genes or tumor behavior remains still to be fully understood. The numerous possible points of histone modification indicate the huge complexity of the system and the very many possible levels of regulation. Methylation of H3 at K4 and K36, as well as acetylation of H3 at K9, are closely linked to transcriptional activation, while the methylation of H4 at K20 is associated with transcriptional repression [36–38]. Therefore, we chose the five histone modification sites in our study to explore the global histone modification patterns to predict the risk of the biochemical recurrence in prostate cancer after RP.

Pound *et al*. [39] reported that no men had

experienced a distant or local recurrence without biochemical recurrence. In order to achieve long-term survival time and disease-free survival time of the prostate cancer following RP, we can firstly assess the risk of the biochemical recurrence on the base of our study, then determine the occurrence of the isolated local recurrence and distant metastasis by analyzing the clinical data such as Ultrasound-guided biopsy, Computed tomography, magnetic resonance imaging, Bone scan, and so on, thus we can give the appropriate treatment-watching and waiting, salvage radiotherapy and hormonal therapy to the patients.

The correlation between the prognosis of the low-grade prostate cancer and the histone modification could be effectively evaluated on condition that the number of the prostate cancer cases in the study was at large. For the high-grade prostate cancer, the analysis of the post-operative prognosis should be accompanied with pre-operation PSA level. However, to completely understand the role of the histone modification in prostate cancer, further studies are still needed.

In conclusion, we have investigated the role of the global histone modifications in prostate cancer, and suggested possible useful prognostic markers for the selection of prostate cancer patients after RP, in terms of the grade of the prostate cancer and the pre-operation PSA level.

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