

The Comparison Between Microwave and Autoclave as Antigen Retrieval Methods for Immunohistochemical Detection of CD15 and CD30 in Hodgkin's Lymphoma

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KEYWORDS

Hodgkin Disease,
Immunohistochemistry,
Microwaves,
CD15 antigen,
CD30 antigen

Article Info

Received 31 March 2017;
Accepted 13 Sep 2018;
Published Online 25 Sep 2018;

ABSTRACT

Background and Objective: Hodgkin's lymphoma is a potentially curable hematologic malignancy with difficulty in its diagnosis especially in atypical cases even in expert hands. Today, immunohistochemistry plays a significant role in the diagnosis of it especially applying the anti-CD15 and anti-CD30 antibodies. The negativity of CD15 can be reduced by antigen retrieval for methods. In this study, the effect of autoclave was compared with microwave as heating sources of antigen retrieval in immunohistochemical staining.

Methods: Sections prepared from 50 formalin-fixed paraffin-embedded tissue blocks of Classic Hodgkin's lymphomas stained for CD15 and CD30 using autoclave and microwave, were randomly and blindly reviewed by an expert hematopathologist, mostly focusing on Reed-Sternberg cells; the intensities were scored from 0 to +4 and analyzed by SPSS software.

Results: Fifty eight percent of patients were male. The mean age was 32 years (range: 7 to 77). Nodular sclerosis was the most prevalent subtype. CD15 positivity in microwave treatment was 92% compared to 50% in autoclave. Negative CD30 decreased from 20% in autoclave to 2% in microwave. Intensity of staining in both markers was at least +1 greater in microwave treatment. No background staining was seen in microwave method.

Conclusion: There was bimodal age distribution in Hodgkin's lymphoma patients with the predominant male and Nodular Sclerosis as the most common type. Comparing autoclave and microwave, higher rate of the positivity was detected using microwave treatment, especially in CD15 staining. Improvement in staining intensity was also noticeable in both markers. There was no background or non-specific staining in microwave method. No disturbance of cells or nuclear morphology was also reported.

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Introduction

Hodgkin's lymphoma (HL) is one of the curable hematologic malignancies which has bimodal age distribution (1-3). Diagnosis of Hodgkin's lymphoma, similar to many other hematologic malignancies, is principally based on the immunohistochemical study of cell surface markers. Even in some cases, flow cytometric and molecular studies are mandatory to avoid misdiagnosis which is not very low (4). The World Health Organization (WHO) 2008 classification schema introduced two histological types of

HL: the nodular lymphocyte predominant (NLPHL) and the "classic" HL (CHL). The latter encompasses four subtypes: nodular sclerosis, mixed cellularity, lymphocyte depletion, and lymphocyte-rich variant. Amongst several enlisted surface markers for diagnosis, CHL, CD15 and CD30 are the most important markers. On the other hand, CD15 is not only a useful diagnostic marker but also has a prognostic effect on patients' outcome (5, 6). Despite all efforts for finding a new fixative, formalin is hitherto, the most popular tissue fixative all around the world (7).

Formaldehyde; however, interacts with tissue and has an adverse effect on proteins and cell surface antigens which leads to weak or negative staining in immunohistochemical (IHC) procedures (8). Accordingly, inconsistent results forced scientists to find suitable "Antigen Retrieval" (AR) methods (9). Enzymatic digestion as an early method of AR was not suitable for many antigens, in addition to difficulty to control the settings (10). Other researches showed that high temperature and strong alkali environment can break formalin-tissue bonds (11). The first article about using heat for antigen retrieval was written by Dr Shi et al. in 1991 (12).

The sources of heating include: water bath, pressure cooker, autoclave, microwave oven

etc. Between these different sources, microwave is interesting because in addition to the ability to rise temperature during heating periods, it has effects on tissue peptides and molecules (13).

Inconclusive results of CD15 staining, regardless of type and clone of antibody used, could be attributed to insufficient heating in AR method and low concentration of primary antibody (14).

In this pilot study, we compared two common methods of heat-induced epitope retrieval (HIER) including microwave oven and autoclave, on the quantity and intensity of CD15 and CD30 IHC staining.

Materials and Methods

Study design

Comprehensive search in major databases like PubMed, Google Scholar, Scopus etc. showed only one similar study, which evaluated 20 Hodgkin cases and but did not report the percent of positivity in CD15 or CD30. Due to the lack of exact statistical data of Hodgkin's lymphoma prevalence in our country, and our estimation of about 25 cases of classic Hodgkin's lymphoma yearly in our center through searching our local database; we designed this pilot study to evaluate the differences in positivity and intensity of CD15 and CD30 staining between microwave oven and autoclave.

Sample selection

We searched our hospital-based pathology reports database for "Hodgkin's lymphoma".

Only the patients with documented diagnosis of classic Hodgkin's lymphoma were included in the study and their subtype was extracted from their diagnostic pathology reports. In the core needle biopsies, which definition of subtype was impossible, they were sorted as unknown subtypes. All cases of Nodular Lymphocytic Predominant Hodgkin's Lymphoma were excluded from the research.

The proper formalin-fixed paraffin-embedded (FFPE) tissue blocks of classic Hodgkin's lymphoma cases with adequate tissue were chosen.

Sample preparation methods

Tissue sections were prepared from formalin-fixed paraffin-embedded blocks. We used primary antibody, secondary antibody, protein block, chromogen, bicarbonate, antibody diluent, Horse Radish Peroxidase enzyme (Envision^{TM+}/HRP, Mo), Diamino benzidine (DAB), washing and blocking buffers, target retrieval solution and Hematoxylin dye from "Dako (international trade mark), Denmark", and immunohistochemistry products. The antibodies against CD15 and CD30 were Monoclonal Mouse Anti-Human clone Carb-3 and Ber-H2, respectively. After routine dewaxing, rehydration, and endogenous peroxidase blocking procedures, the slides were transferred to plastic Coplin jars containing the special target retrieval solution of antibody. Each tissue section from the same FFPE blocks, pretreated with autoclave at 134°C for 10 min and also with Butane (local trade mark) microwave oven with maximum strength of 900 watts for 2 min and then with 400 watts for another 8 min. Other steps including adding protein block, primary and secondary antibodies, chromogen, drying and mounting the slides were the same for both methods. Fifty cases were stained for CD15 and CD30 with autoclave and microwave methods. Two hundred immunohistochemically stained slides were randomly and blindly reviewed by a Hematopathologist (One of the authors). The positive staining was only accepted in Reed-Sternberg cells. The pattern of

staining was submitted as Golgi Zone, Membranous, Cytoplasmic and combination of these patterns. The intensity of staining was scored as:

Negative: no staining

1+: hardly visible

2+: barely visible with some attempts

3+: easily visible in low power (x40)

4+: very easily visible and very strong staining in low power

The demographic data of patients, subtypes of Hodgkin's lymphoma and the score of staining were gathered and analyzed.

Statistical analysis

Continuous variables were described with mean \pm SD; and categorical variables were expressed as frequency with percentage. The association between ordinal variables was assessed using Spearman correlation coefficient and gamma measure of association.

For nominal data, kappa or Cramer's V measures of association were used. All statistical description and analyses were conducted using IBM SPSS statistics for Windows version 23.0 (Armonk, NY: IBM Corp.).

Results

Demographic characteristics

Among patients, 29 (58%) were male and 21 (42%) were female. The mean age of patients was 32 (min: 7 and max: 77) years old. The most common subtypes of Hodgkin's lymphoma were Nodular Sclerosis (NS) (50%) and Mixed Cellularity (26%). In both types, male patients were more than females. The largest number of patients was in 20-40 years age group as illustrated in Table 1. Hodgkin's lymphoma has a bimodal age which is mostly seen in 20-40 (48%) and then 40-60 (26%) years old. Both markers had almost uniform staining pattern and intensity in each slide examination.

Table 1. Frequency of Hodgkin subtypes in different age groups

		Age grouping (years old)					Total No (%)
		<10	10-20	20-40	40-60	>60	
Hodgkin types	NS	2	4	13	5	1	25 (50)
	MC	1	3	2	6	1	13 (26)
	Unknown	0	1	9	2	0	12 (24)
Total		3 (6%)	8 (16%)	24 (48%)	13 (26%)	2 (4%)	50 (100%)

CD15 staining in autoclave and microwave

Positive staining of CD15 was 50% in autoclave method which increased to 92% in microwave method. The intensity of staining in autoclave method was 1+ and 2+ in about 40% of cases versus 2+ and 3+ in 70% of cases in microwave processing. Detailed percentages of each score staining are summarized in Table 2. Golgi zone pattern staining was the same in both methods (26%), membranous pattern increased fourfold in microwave staining (48% vs 12%). Other Golgi zone including patterns (Golgi and cytoplasmic, Golgi and membranous, Golgi and cytoplasmic and membranous) increased in microwave (18% vs 12%).

CD30 staining in autoclave and microwave

Positive staining of CD30 was 80% in autoclave method which increased to 98% in microwave method. Intensity of staining in autoclave was mostly 1+, 3+ and 4+ versus mostly 2+, 3+ and 4+ in microwave method. None of the cases were 1+ (hardly visible) in microwave processing. Detailed percentages of each score staining are summarized in Table 2. Golgi zone pattern staining was 14% in autoclave and 6% in microwave; membranous pattern increased 26% in microwave staining (48% vs 74%). Other Golgi zone including patterns (Golgi and cytoplasmic, Golgi and membranous, Golgi and cytoplasmic and membranous) were the same in both methods (18%).

Examples of microwave staining intensity score are shown in Figure 1.

Paired CD15 and CD30 Staining pattern

For comparing the paired staining pattern in each case of Hodgkin’s lymphoma, the cases were divided to four groups as below:

Group A: CD15 + and CD30 +

Group B: CD15 + and CD30 -

Group C: CD15 - and CD30 +

Group D: CD15 - and CD30 -

The rate of double positive markers Hodgkin’s lymphoma cases (Group A) increased from 44% in autoclave to 90% in microwave method. One marker negative (Groups B and C) rate also decreased in microwave. Double negative pattern (Group D) was not observed in microwave staining. More details of groups A-D are summarized in Table 3.

Table 2. Intensity of CD15 & CD30 staining in autoclave and microwave methods

Intensity	CD15 (%)		CD30 (%)	
	Autoclave	Microwave	Autoclave	Microwave
+	28	18	18	0
++	12	42	6	18
+++	8	28	16	50
++++	2	4	40	30

Table 3. Pair pattern of staining of CD15 and CD30 in Autoclave and Microwave methods

Staining Pattern	Autoclave (%)	Microwave (%)
A (CD15+, CD30+)	22 (44)	45 (90)
B (CD15+, CD30-)	3 (6)	1 (2)
C (CD15-, CD30+)	18 (36)	4 (8)
D (CD15-, CD30-)	7 (14)	0 (0)

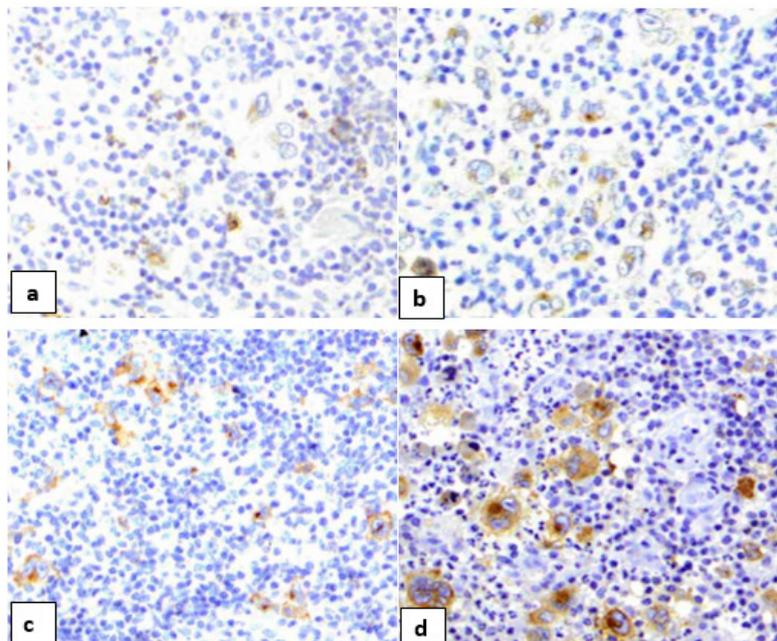


Figure 1. One to four plus staining in microwave heating antigen retrieval method, a to d, respectively in high power field (×40).

Analogy of autoclave and microwave

The mentioned methods are heat-treating methods of Antigen Retrieval (AR). Scoring of them was ordinal. Therefore, we compared them with Gamma and Spearman correlation factor. Gamma and Kappa were 0.043 and -0.177 with Spearman correlation factor of -0.14 for comparing CD15 in autoclave and microwave staining. For CD30, Gamma was -0.73 with Spearman correlation factor of -0.075 for autoclave versus microwave. These correlation factors showed that these two methods were different and had little correlation with each other.

Discussion

We found male predominance in classic Hodgkin's lymphoma patients with male to female ratio: 1.38/1 which is in concordance with the previous literature (1, 15, 16). The mean age of our patients was 32 years. We observed the bimodal pattern of Hodgkin's lymphoma as noted in the reference hematology books and other researches; however, our later life peak was about two decades lower than other geographical areas (1-3, 15, 16). Nodular Sclerosis (NS) was the most common subtype of classical Hodgkin's lymphoma in our patients and Mixed Cellularity (MC) was in the second place. This prevalence pattern was seen in many other countries, only in one study in India, the Mixed Cellularity was more common than NS (1, 3, 16, 17). Nodular Sclerosis has been seen in young women, but we had more in young men which was similar to the Indian study (1). Percentage of CD15 negativity in Hodgkin's lymphoma was different in various studies; ranges between 15 to 40% (1, 4, 17-19). In our routine clinicopathological laboratory procedure (using autoclave heating), our CD15 negative results was more than other researches. In other words, we had large percent of CD15 negativity like pre-AR era (19). In review of this case, we found that in autoclave method, most of the cases were 1+ and 2+ and almost all cases of 1+ were Golgi zone staining pattern, which mandates a comprehensive study to be seen. One reason of our sizable negative percentage could be attributed to negligence of pathologist during review of IHC slides. Although CD15 staining is

generally difficult and gives weak staining most of the times (18); it shows strong staining using microwave as AR method. Granulocytes showed strong staining in this method as internal control. The CD30 is easier to be performed than CD15 (18). Generally, the rate of CD30 positivity in Hodgkin's lymphoma ranges from 93.5 to near 100% with recent improvement in IHC techniques (1, 4, 17, 19). We had acceptable percentage of positivity, even in autoclave method. However, the quality and quantity of staining increased in microwave method up to 98% in the cases. Moreover, the amount of hardly visible (1+) cases reduced to zero in microwave treatment, and the percent of 2+ and 3+ staining increased about threefold. Similar to the study of Charalambus et al. we can recommend the use of microwave in AR especially in regards to CD15 and CD30 staining in Hodgkin's lymphoma (20). Our results are in keeping with Cuveas et al. study which showed microwave retrieval method was beneficial for long list of antibodies including CD15 and CD30; which also has the advantage of using higher dilutions of antibodies (21).

Some studies suggest that Golgi and cell membrane staining patterns are more specific for Reed- Sternberg cells (4). Some studies note that dot-like Golgi zone and membrane patterns are specific for lymphoid neoplasms (17). In our study, Golgi zone pattern in CD15 with autoclave method was the commonest one. The intensity of various patterns including Golgi zone pattern has increased by microwave treatment. Membranous pattern was the commonest pattern in CD30 staining in both methods and other patterns including Golgi zone were similar in both methods. Despite some studies which claimed about microwave disadvantages (22), we did not have non-specific background staining, diffuse non-diagnostic staining or any confounding distortion of cellular details or morphology.

In evaluating the paired staining pattern of CD15 and CD30 in classic Hodgkin's lymphoma cases, we found double positive markers as commonest form of patterns like other studies (1, 19). The percent of this pattern (Group A as mentioned earlier)

ranges between 60 to 83% in studies, compared to 44% and 90% in autoclave and microwave methods, respectively in our study (1, 19). It seems that using microwave we could achieve more acceptable range of double positive marker in IHC study of Hodgkin's lymphoma cases. The CD15 - & CD30 + (pattern C) decreased from 36% in autoclave to 8% in microwave treatment compared to 16% in Von Wasielewski et al. study which was in favor of stronger impact of microwave heating on CD15 retrieval than CD30 (19).

Conclusion

It was concluded that microwave AR method have more positive staining rate in both CD15 and CD30, especially CD15. Furthermore, the intensity of staining increased at least one plus more than autoclave treatment which results in easier, faster and more accurate diagnosis of Hodgkin's lymphoma cases without any false positive results, non-specific staining or histomorphologic distortion. This is crucial for patient management, as CD15 is considered not only a diagnostic but also a prognostic marker. Microwave is

an inexpensive, available, easy to standardize, and user-friendly device with the mentioned beneficial AR effects on many antigens used in IHC staining. Today, the microwave treatment has also been applied for the extraction of proteins and nucleic acids from tissue samples in many recent molecular and proteomic methods.

This pilot study was found to present satisfying results about microwave tissue treatment, which could be used as a basis to design larger studies concerning different antibodies as well as multiple variables like temperature, buffer solutions, time intervals and different PH values.

Acknowledgment

This research was supported by Tehran University of Medical Sciences & Health Services grant No: 28309.

Conflict of interest

The authors declare that there is no conflict of interest.

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How to Cite This Article

Kosari F, Ghaffari F. The Comparison Between Microwave and Autoclave as Antigen Retrieval Methods for Immunohistochemical Detection of CD15 and CD30 in Hodgkin's Lymphoma. *Iranian Journal of Pathology*, 2018; 13(4): 390-396.