Touch Imprinting Cytology may be useful in the intraoperative evaluation of the sentinel lymph node in melanoma

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PURPOSE: *The aim of the study was to assess whether the reliability of Touch Imprinting Cytology (TIC) of Sentinel lymph node biopsy (SLNB) in skin melanoma patients allows intraoperative decisions regarding simultaneous radical lymphadenectomy to be made. Previous experiences have shown that the limit of TIC in extemporaneous diagnosis was represented by the minimal deposits of the tumor. Many current data seem to show that in this situation radical lymphadenectomy is no longer necessary, so we wondered if TIC could regain importance in the intraoperative management of these patients.*
METHODS: *TIC results of Sentinel Lymph Nodes Biopsy (SLN) were compared with those of standard histopathological and immunohistochemical examinations.*
RESULTS: *A total number of 110 SLN were detected from 50 melanoma patients.TIC revealed the presence of metastases only in 1 out of 13 melanoma-positive SLN (sensitivity 7.6%). There were no false-positive results of TIC (specificity 100%). The negative predictive value was 75.5%, the positive one 100% with a total diagnostic accuracy of 76%.*
CONCLUSIONS: *TIC for SLNs is a reliable method, relatively fast and not very expensive. Although with a very high specificity, its sensitivity was very low, and almost exclusively limited to macro-metastases (>2mm). Furthermore, it was not possible to identify a subgroup of patients, based on the characteristics of the primary tumor, in which the method could have been more useful. Finally, even in positive cases, the method rarely reduced the need of a tactic in two stages, principally for the management of the operating room.*

KEY WORDS: Melanoma, Sentinel lymph node (SLN), Touch Imprinting Cytology (TIC)

Introduction

Sentinel lymph node biopsy (SLNB) introduced by Norton for melanoma in 1992 is now widely accepted as a highly accurate method for assessing regional lymph nodes (> 95%) [1-17]. The knowledge of the lymph node involvement has a precise prognostic significance and it represents an important indication for a Complete Lymph Node Dissection (CLND) [1-5,16-17]. The pathological evaluation of SLNs is, however, very hard and complicated, as melanoma metastases can also be represented by a few isolated cells with unspecific morpho-structures that require immunohistochemical stain to correctly interpret them. It is therefore a long process requiring preventive fixation in formalin and the study of multiple sections after specific colorings.
Today CLND is expected for patients with positive SLNs. It is a 2-stroke tactic, with 2 interventions on the same operative site at a short distance. To overcome this problem, some authors have suggested an extemporaneous frozen analysis of the SLNs, with or without

Pervenuto in Redazione Marzo 2019. Accetttao per la pubblicazione Aprile 2019
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immunohistochemistry [3-5,12]. This approach has been strongly criticized both for a low sensitivity (on average lower than 30%) and for morphological alterations and loss of tissue during the slide preparation that can prejudice the histological diagnosis.
For these reasons, some studies have appeared in literature about the possibility of performing an extemporaneous TIC analysis on SLN melanoma. In these early experiences, TIC, despite its very high specificity [1,4,5,16], showed a very variable sensitivity (from 30 to 63%) [1,5,6,8,9,10,11,13,16] largely related to the size of lymph node metastases. Furthermore, TIC has proven to be a relatively fast and cheap method (about 1/3 compared to the frozen test), but it requires dedicated staff with specific cytological abilities.
Starting from these premises, the main purpose of this study was to evaluate TIC sensitivity and specificity in the extemporaneous diagnosis of SLN metastases. Furthermore, we wanted to evaluate the impact of this method on the clinical management of the patient, in particular the possibility of reducing the 2-stroke tactic. As a secondary objective, we sought to identify a possible subgroup of patients for whom the method might have been particularly useful.

Patients and Methods

Since 2010, 360 patients had undergone a SLNB and radicalization of the surgical wound for a trunk and limbs melanoma at the Institute of General Surgery of the University of Trieste. In the last 50 patients, TIC analysis was performed prospectively on all SLNs for the evaluation of the presence of lymph node metastases. The TIC results were compared with those derived from standard histopathological and immunocytochemical examination. All patients prior to surgery were subjected to a careful clinical examination and to ecotomography of the lymph node districts to exclude the presence of metastates. In this latter case, after cytology confirmation, patients were directly submitted to a CLND. All patients with advanced neoplasia (T3-T4) performed a complete staging with a TC Total Body or Pet with FDG. The site and stage of primitive neoplasia are listed in Table I. There were 27 males and 23 women (average age 48). We found no significant differences between sex and the position of the neoplasm (p = 0.100) and between sex and thickness of the lesion measured according to Bresslow (p = 0.507). Similarly, we found no significant differences between the site of melanoma and its thickness and between site and ulceration, although trunk neoplasms were more frequently ulcerated (respectively 27.3% vs 32.1%).
The present retrospective cohort study was evaluated and approved by the UNIQUE Ethical Committee of the Friuli Venezia Giulia Region with the number 167_2018.

Surgical Technique

In most cases the intervention was performed under general anesthesia.
For the sentinel lymph node research we used almost exclusively lymphoscintigraphy with nanocolloids containing Tc99. At the discretion of the surgeon, in some cases, a technique combined with associated vital dye was used. When possible, the SLN research was performed as initial phase of the surgical procedure, so that the time required for TIC evaluation was used for the "radicalization" of the cutaneous exeresis. This allowed us to minimize the impact of TIC on operating time.
All "hot" lymph nodes (those with a greater radioactivity than the basal one, conventionally fixed at 10% of the radioactivity found in the hottest lymph node) were removed and evaluated.
All patients with limb melanoma presented a single lymphatic, axillary or inguinal drainage pathway. In the 28 patients with trunk disease, lymphatic drainage occurred in 20 cases in a single basin, in 5 cases with 2 basins, and in the last 3 cases with 3 basins.
In all patients, we successfully detected and removed the SLNs along the main drainage path (defined as the first and most visible after the injection of scintigraphic contrast). Only in 3 cases, with trunk melanoma, it was not possible to locate SLNs along a secondary drainage pathway. Although the SLN of the "main" site was negative, these patients were inserted into a more intensive follow-up.
The sucessful rate in SLNB for the main drainage pathway was 100%, while for all lymph nodes detected with pre-operative lymphoscitigraphy was 96% (61/64 basins).

Table I - *Relationship between site of primary melanoma and T stage (p = 0.451)*

Location	Cases	T1a	T1b	T2a	T2b	T3a	T3b	T4
Upper limbs	3 (8%)	0	1	0	0	2	0	0
Lower limbs	19 (38%)	1	3	7	3	2	2	1
Trunk	28 (54%)	1	7	13	5	2	3	1
Total	50	2	7	20	8	6	5	2

Table II - *Relationship between site of primary melanoma and number and condition of SLNs*

Location	Cases	N° districts involved	N° lymph nodes found	N° metastatic lymph nodes	N° metastatic patients
Estremity	22	24	46	7	4
Trunk	28	37	64	10	9
Total	50	61	110	17	13

TABLE III - *Relationship between primary site of melanoma and site, number and condition of SLNs.*

Location Primary melanoma	Cases tot.	Sentinel lymph node (SLN) Axillary Tot./n+cases	Inguinal Tot./n+cases	Axillary and Inguinal Tot./n+ cases
Estremity	22	3/2	19/5	0
Trunk	28	20/4	4/1	4/1 (Axilla)
Total	50	24/6	22/6	4/1

In these 50 patients 61 drainage basins were explored with a total of 110 SLNs (range 1-5) and an average of 2.2 SLNs per patient (Tables II and III).
No statistically significant correlation was observed between the number of removed SLNs and the T stage and ulceration of tumor (respectively p = 0.23 and p = 0.81).

PATHOLOGICAL EXAMINATION

All removed SLNs were subjected to TIC. For this purpose they have been bisected along the major axis, paying great attention in order to obtain complete cross sections of the maximum size, preferably including the hilum and the marginal sine.
For each half of the SLN, a couple of impressions were made by gently touching the SLN cutting surface on a slide. The impressions of each surface were immediately fixed in 95% ethanol for 3 minutes and then stained with hematoxylin and eosin (H & E). The cytological smears were examined by a pathologist, a diagnosis of positive or negative outcome for tumor was made and notified to the surgical team.
Subsequently, the SLN was fixed in 10% formalin, processed as usual and embedded in paraffin. An initial H & E section of each half of the SLN was cut from the paraffin block. If the initial revision of the stained sections with H & E was negative, the melanoma protocol included 3 additional H & E staining levels cut at 150 μm intervals in combination with immunohistochemical stains for S-100 and HMB-45 on each of the 3 levels. Immunohistochemical studies were performed using the automatic autostainer (BenchMarrk Ultra Ventana). Primary antibodies included S-100 (Policlonal Ventana) and HMB-45 (Ventana antimelanosoma monoclonal antibody). Immunohistochemical stains were considered positive for MM if the immunoreactivity was detected in groups of cells or single cells that demonstrated anatomical and cytological features of metastatic tumor cells. The average time for TIC was around 30 minutes.

STATISTICAL ANALYSIS

TIC results were compared with standard histopathological and immunohistochemical results, which were considered as reference results. Sensitivity, specificity, positive and negative predictive value and TIC accuracy were calculated.
Statistical analysis was done using SPSS (Package Statistical for Social Science). Categorical variables were reported as percentages and frequencies, while continuous variables were reported as mean, median, and standard deviation.

TABLE IV - *Relationship between characteristics of primary melanoma and frequency, number and type/size of SLNs metastases.*

Primary melanoma	Tot cases	Patients with positive SLN	Analyzed SLN	N+SLN	Pathological aspect of metastatic lymph nodes: ITC or micro metastases (< 0,2mm)	Metastases >0,2 e <2mm	Metastases > =2 mm
T classification (AJCC 2009)							
T1	9	1 (11.1%)	18	1	1	0	0
T2	28	7 (25.0%)	61	7	4	1	2
T3	11	4 (36.4%)	25	7	2	0	2
T4	2	1 (50.0%)	6	2	0	1	0
P (Chi Square Pearson)		P= 0.513	P= 0.513				
Ulceration							
Present	15	6 (40%)	36	7	4	1	2
Absent	35	7 (20%)	74	10	3	1	2
P (Fisher's exact test)		P=0.170	P=0.170	P=0.194			
Site							
Estremity	22	4 (18.2%)	46	7	2	1	1
Trunk	28	9 (32.1%)	64	10	5	1	3
P (Fisher's exact test)		P=0.339	P=0.339	P=0.265			

TABLE V - *Method of diagnosis in relation to the size in the 13 Metastatic SLNs*

Size of lymph node metastasis	Cases	Diagnosis		
		H&E	Immune histochemical	Touch imprinting
ITC e Micromet. < 0,1 mm	4	0	4	0
Micrometastasis 0,2 -2mm	2	0	2	0
Metastases 1- 2 mm	3	1	3	0
Metastases > 2 mm	4	4	4	1
Total	13	5	13	1

For the comparison of categorical and continuous variables between different groups, we used the chi-square test and the ANOVA. A p value of less than 0.05 was considered statistically significant.
Sensitivity, specificity, positive and negative predictive value and TIC accuracy were determined on a patient basis.

Results

LN metastasis occurred in 13 patients (26%): in 12 cases the metastasis was localized on the first SLN (the one with a higher gradient of radioactivity) while in one case it concerned only a lymph node with a minor radioactive gradient. Contemporary metastatic involvement of multiple SLNs occurred in 3 cases. The number and type of lymph node metastasis (ITC, micro-metastasis or macro-metastasis) in relation to characteristics of primary melanoma and number of lymph nodes analyzed are shown in Table III.
The TIC sensitivity to identify SLN metastates was therefore 7.6% with a specificity of 100%. The negative predictive value was 75.5%, the 100% positive one with a total diagnostic accuracy of 76%.
Regarding the size of the lymph node metastasis (which was not detected preoperatively with ecotomography), in 4 cases it was macro-metastasis (diameter> 2 mm), in 5 cases micro-metastasis (diameter <2 mm) and in the last 4 cases single tumor cells or small clusters. The relationship between metastasis dimension and method of diagnosis is shown in Table IV.
The TIC sensitivity was related to the size of metastasis, with a 25% positivity for macro-metastasis and 0% in case of micro-metastasis or 0% isolated cells.
The only case in which TIC was positive for an inguinal SLN, for organizational reasons, it was not possible to proceed directly to CLND, which was rescheduled after a few days.

Discussion

It is well known that in patients with melanoma, the lymph node situation assumes a precise meaning both prognostic and therapeutic [18]. The SLNB with a low morbidity/mortality rate, can predict, with more than 95% accuracy, the lymph node regional situation [1-12]. Thanks to this method it is possible to provide the pathologist with one or more lymph nodes for very accurate analyses. These are, moreover, complex and require a lot of time and dedicated staff, and they include immunohistochemical investigations. Along with these traditional surveys, today molecular biology can also be performed to find RNA messenger of tyrosinase with methods of RT-PCR. This technique, compared with the conventional histopathology evaluation, allows a further refinement of the investigation even if, due to the presence of capsular nevus cell agglomerates in 10% of the lymph nodes, it may lose specificity. Nowdays, the anatomopathologist is able to find even a single metastatic cell in SLNs, but the prognostic/therapeutic significance of these minimum tumor deposits is not clear [19.21].
In fact at present, the benefits of CLND after positive SLNB have been confirmed by many works, but above all by the 10-year final results of the MSLT I (International Multicenter Study) [18] which randomized patients with intermediate thick melanomas (from 1,2 to 3,5 mm) for SLNB (60%) or for simple observation (40%). In this work N + patients undergoing SLNB and early CLND exhibited a lower local recurrence rate and above all a free disease interval and a significantly better specific survival rate. These data support recommendations to perform SLNB and a CLND after each positive SLNB. This is a 2-stroke tactic, which includes the discomfort of a second general anesthesia at a short distance. This strategy, which also involves repeated tissue manipulation, has been screened in some studies as a possible cause of increased incidence of local infections/lymphedema and nerve injuries, but also of a local spreading of neoplastic cells, which may affect a higher incidence of local recurrences [14].
It was therefore attempted to assess the situation of the SLNs in the operating room but all attempts made so far have been unsatisfactory, especially because of their low sensitivity. In details, the frozen sectioning, which is recognized as sensitive to minimum neoplastic deposits in 30% of cases [3-5,12], is currently strongly discouraged, both for alterations and loss of tissue during preparation which may compromise the evaluation of that lymph node [5,6,10].
By borrowing the experience of breast cancer, a number of works with TIC evaluation in melanoma patients has been published. Early TIC studies suggested that this technique could be used to confirm intraoperatively the presence of metastasis in macroscopic pathological SLNs, with a sensitivity of 62% without false positives (5.7). In centers where TIC analysis was performed freely in all patients, the results were mostly homogeneous and not very encouraging with a sensitivity varying from 21% to 47% [1,2,5,6,8,10,13] although with a specificity close to 100%. It is therefore evident TIC has a greater sensiti-

vity in patients with significant lymph node tumor deposit rather than micro-metastases: in Soo's work [13] TIC sensitivity for macro-metastases detection (>2mm) was 62% vs 16% for micro-metastases (P<.01) in Hocevar's [10] respectively 78% and 14% (p> .01).

A new interest for TIC can, in our view, derive from the latest studies on the significance and indication for early CLND in patients with a minimum tumor SLN deposit. Indeed, these studies (in particular the MSLT II randomized multicenter study) [19-21] show that in these patients early CLND would not add any benefit.

If these studies are confirmed by further experiences, patients with minimal cancer SLN deposit (where TIC has shown low sensitivity) won't require an early CLND anymore. As a consequence we opted for this prospective study, with the aim of verifying whether TIC might have reduced the need for a 2-stroke surgery.

In our experience, TIC sensitivity was considerably lower than reported in literature, with only one positive TIC case in 13 N + patients (7.7%) and 1 out of 7 patients with metastases> 1 mm (14.3%).

In our experience the careful preoperative lymph node ultrasound staging, associated with FNA, allowed us to directly identify macro-metastases and this may have influenced both the low TIC sensitivity and the incidence of macro-metasteses.

Finally, in the review of our false negatives, we found that none of them (even when bigger than 2 mm) was on cutting surface. However, the recognition with a cytological examination of melanoma metastases is often very difficult so the differential diagnosis can be made only with immunohistochemistry.

In our analysis, as well as others [5,8,9], the absence of any significant correlation between the characteristics of primitive melanoma and dimension of lymph node metastasis emerged if the lymph node preoperative ultrasound staging was negative. Therefore, it wasn't possible to select a subgroup of patients with a higher risk of lymph node macro-metastasis for which a TIC might indeed have had a greater sensitivity. On the other hand, this aspect was evaluated by Creager [1] who noted an increase in lymph node macro-metastases parallel to the increase of thickness of the primitive tumor.

TIC analysis was also demanding because the analyzed SLNs were often more than one (in our experience 2.2 SLNs per patient) therefore requiring a longer surgical intervention.

TIC benefits are also very modest considering that patients with primary melanoma metastases are 20-25% [1-8,18] whereas those >1 mm are about 40% [1,5,18,21]. As the sensitivity of the method for metastasis of this size or greater was 30% [1,2,5,6,8,10,13], patients who could benefit from TIC would not be more than 3-4% of cases.

Finally, not all patients with positive TIC could be directly subjected to CLND, for flexibility/planning of the operating room. In fact, while an axillary CLND is relatively rapid and can easily be performed even without a previous program, a long-lasting inguinal or cervical lymph node dissection can rarely be managed directly. This consideration would therefore limit the indication for TIC only to patients with axillaries SLN.

A further problem is that of false positives that can lead to the execution of an unjustified CLND [1-4-19-21]. In most studies, however, TIC specificity appears to be 100% even though in Hocevar's work, out of 99 patients and 215 analyzed lymph nodes, 3 patients showed falsely positive TIC. [10].

All these considerations led us, after the experience with the first 50 patients, to suspend TIC analysis in SLNB. Today, in the attempt to reduce a second CLND, we enhanced the preoperative LNs staging, both with the help of dedicated ultrasound personnel and of FNA in case of morphological/structural alterations and vascularization of LNs.

Conclusions

The aim of this study was to evaluate TIC impact on management of SLNB candidates for melanoma, considering recent studies that do not confirm the advantages of immediate CLND in case of micro-metastasis/ITC. In fact these 2 situations were more difficult to diagnose with TIC.

In our experience, which involved only N0 patients in preoperative staging, TIC sensitivity in the diagnosis of SLN metastases was 7.5%, therefore lower than that reported in literature [1-10], and was related to the size of metastases (with a sensitivity of 25% for lesions greater than 2 mm).No correlation was found between TIC sensitivity and the characteristics of primary tumor (Breslow thickness, mitosis, site and stage T). Therefore, it was not possible to identify a subgroup of patients, based on the melanoma characteristics, with a more sensitive TIC.

Our study confirmed that TIC is a relatively complex method, requiring time and highly specialized staff and inevitably influencing surgery length. However, by evaluating the incidence of melanoma LN metastasis and TIC sensitivity, those who may have an advantage from TIC (avoiding a second intervention), would be no more than 3-4%. Furthermore, for reasons of flexibility/planning of most operating theaters, after positive TIC it is possible to directly execute a CLND only for axillary site.

Therefore, at present, we believe that routine TIC performance in SLN melanoma does not result in any benefits at the expense of cost increase, surgery length and a potential risk of having false positives and therefore unjustified CLND. All these considerations have induced us to suspend TIC analyses, therefore favoring an accurate preoperative LN staging with ultrasonography and FNA.

Riassunto

Lo scopo dello studio era di valutare se l'affidabilità di Touch Imprinting Cytology (TIC) nella biopsia del linfonodo sentinella (SLNB) nei pazienti con melanoma cutaneo consente di prendere decisioni intraoperatorie riguardanti la linfoadenectomia radicale simultanea. Precedenti esperienze hanno mostrato che il limite del TIC nella diagnosi estemporanea era rappresentato dai depositi minimi del tumore. Molti dati attuali sembrano mostrare che in questa situazione non è più necessaria la linfoadenectomia radicale, quindi ci siamo chiesti se il TIC potesse riacquistare importanza nella gestione intraoperatoria di questi pazienti.

I risultati TIC della biopsia del linfonodo sentinella (SLN) sono stati confrontati con quelli degli esami istopatologici e immunoistochimici standard: su 50 pazienti con melanoma sono stati prelevati un totale di 110 SLN, e su questi è stato individuata la presenza di metastasi solo in 1 su 13 SLN melanoma-positivi (sensibilità 7,6%), senza falsi positivi di TIC (specificità 100%). Il valore predittivo negativo era del 75,5%, quello positivo del 100% con una precisione diagnostica totale del 76%.

In conclusione la TIC per SLN è un metodo affidabile, relativamente veloce e non molto costoso. Sebbene con una specificità molto elevata, la sua sensibilità risulta molto bassa e quasi esclusivamente limitata alle macrometastasi (> 2mm). Inoltre, non è stato possibile identificare un sottogruppo di pazienti, in base alle caratteristiche del tumore primario, in cui il metodo avrebbe potuto essere più utile. Infine, anche in casi positivi, il metodo raramente ha ridotto la necessità di una tattica in due fasi, principalmente per la gestione della sala operatoria.

Queste considerazioni ci hanno spinto a sospendere l'analisi TIC intraoperatoria a favore di una più accurata ecografica dei linfonodi superficiali ad opera di personale dedicato, da eseguirsi prima di ogni ricerca del linfonodo sentinella.

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