Ann Surg Oncol (2015) 22:1950–1958
DOI 10.1245/s10434-014-4199-z

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – MELANOMAS

Pathologic Complete Response to Intralesional Interleukin-2 Therapy Associated with Improved Survival in Melanoma Patients with In-Transit Disease

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ABSTRACT

Purpose. Melanoma patients with in-transit disease have a high mortality rate despite various treatment strategies. The aim of this study was to validate the role of intralesional interleukin (IL)-2, to understand its mechanism of action, and to better understand factors that may influence its response.

Methods. We retrospectively collected the clinicopathological data of 31 consecutive patients who presented to a tertiary care cancer center for treatment of in-transit melanoma with intralesional IL-2. Kaplan–Meier survival curves and multivariable Cox regression analysis were performed. Immunohistochemistry (IHC) was used to better understand the immune response to localized IL-2 therapy. Targeted next-generation sequencing was performed to genomically characterize the tumors.

Results. Ten patients (10/31, 32 %) achieved a pathologic complete response (pCR), 17/21 (55 %) had a partial response, and 4/21 (19 %) had progressive disease on treatment. pCR to IL-2 therapy was associated with overall survival (log-rank $p = 0.004$) and improved progression-free survival (PFS) [adjusted hazard ratio (HR) 0.11; 95 % CI 0.02–0.47; $p = 0.003$). A higher CD8+ T cell infiltrate was identified in in-transit lesions with a pCR compared with the other lesions (mean IHC score 3.78 vs. 2.61; $p = 0.01$). Patients with an elevated CD8+ infiltrate demonstrated an improved PFS (unadjusted HR 0.08; 95 % CI 0.01–0.52; $p = 0.008$).

Conclusions. Thirty-two percent of patients achieved pCR with intralesional IL-2 therapy and had a significantly improved PFS compared with the rest of the cohort, which may be explained by a systemic CD8+ T-cell response.

In 2014, it is estimated that there will be 76,100 new patients diagnosed with melanoma in the US, and 9,710 patients will die from the disease.[1] Melanoma has a unique pattern of locoregional recurrence known as in-transit metastases, which occurs in 5–20 % of melanoma patients.[2,3] In-transit metastases develop in the skin and subcutaneous tissues more than 2 cm from the primary lesion but not beyond the regional nodal basin.[4] The 5-year survival of melanoma patients with in-transit disease ranges from 12 to 69 %.[5–8]

There are many therapeutic strategies for in-transit disease, including surgical resection, radiation, regional chemotherapy via isolated limb infusion or perfusion, localized injection of immune-modulating agents, or

First Received: 27 August 2014;
Published Online: 1 November 2014

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systemic therapies.[5,9] Isolated limb perfusion has short-term response rates ranging from 25 to 81 %, with significant adverse effects, including limb amputation, skin or soft tissue damage, deep vein thrombosis, compartment syndrome, or prolonged hospital admission.[10–13] Isolated limb infusion is a less invasive method but with short-term response rates between 23 and 44 %, and complications such as limb amputation, deep vein thrombosis, and compartment syndrome.[12,14–17]

Another well-described approach is localized injection of a therapeutic agent at the site of in-transit lesions.[18] Various immune-modulating agents have been used as intralesional therapy, which are easily administered in the office. Forty years ago, Donald Morton introduced Bacille Calmette–Guérin (BCG).[19] Despite having a high local response rate of up to 90 %, intralesional BCG caused significant toxicities such as disseminated intravascular coagulation, anaphylaxis, and death, resulting in a significant decline in its use.[20] Topical imiquimod and intralesional interferon-α/β have also been reported in either small case-series or in combination with other agents, making it difficult to disentangle the true impact of these agents.[20–23] Several studies have also reported the use of Proleukin® (aldesleukin, Novartis, Quebec, Canada), an analog of human interleukin (IL)-2. Short-term response rates range from 25 to 96 %, and its major advantage is low-grade toxicity.[24–28]

A better understanding of factors that modulate response to IL-2 therapy is needed. For instance, oncogenic *BRAF* has demonstrated immunosuppressive properties,[29,30] and therefore *BRAF*V600E or other mutations may impact response to IL-2. In addition, two studies have suggested that intralesional IL-2 may influence survival; however, the association of response to IL-2 with survival in an independent manner has not yet been reported.[24,28,31] Furthermore, the immune mechanism of intralesional IL-2 therapy has not been well studied in in-transit disease. T-cell-rich infiltrates and an increase in circulating CD8+ T-cells have been reported in only a few patients.[26,28,32] Therefore, studying a larger cohort of patients will assist in elucidating the mechanism of intralesional IL-2 in melanoma patients.

In order to better establish the localized and long-term response to intralesional IL-2 therapy, we performed a retrospective review of melanoma patients with in-transit disease treated with intralesional IL-2 since 2009 at Sunnybrook Health Sciences Centre, Toronto, ON, Canada. To better understand if response to IL-2 therapy can be modulated by tumor-derived mutations, targeted next-generation sequencing of 700 mutation hotspots was performed for the first time in this patient cohort. Furthermore, to better understand the mechanism of action of intralesional IL-2, we tested a panel of immune-mediated markers on prospectively-collected tissue specimens post-IL-2 treatment.

MATERIALS AND METHODS

Study Population

With institutional Research Ethics Board approval, a retrospective review of a single surgeon's (FCW) prospective database of melanoma patients with in-transit disease treated with intralesional IL-2 at Sunnybrook Health Sciences Centre was completed. Baseline characteristics and follow-up data were obtained from the electronic patient records and by contacting referring doctors.

Interleukin (IL)-2 Treatment Protocol

IL-2 was prepared by the pharmacy as 5 million IU/mL. The maximum dose per cycle was ≤20 million IU per cycle, with the volume of IL-2 injected into each lesion varying with the size of the lesion.[24,33] All in-transit lesions were injected intralesionally for one cycle every 2 weeks. Biopsies of lesion(s) were performed 8 weeks after completion of treatment to assess the type of response. Pathologic complete response (pCR) was defined by the absence of histologic evidence of residual disease.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin-embedded tissue blocks collected 8 weeks post-cessation of IL-2 treatment from biopsies of in-transit lesions from 18 patients. IHC was performed as per the Pathology Research Program, Toronto General Hospital, Toronto, ON, Canada. Ten immune markers were utilized: CD4 (NCL-L-Cd4-368, Leica Biosystems); CD8 (VP-C324, Vector Laboratories); CD30 (M0751, Dako); CD20 (M0755, Dako); CD68 (M0876, Dako); CD3 (A0452, Dako); CD1a (IM1590 CC1, Beckman Coulter); CD56 (156R-97, Cell Marque); CD138 (M7228, Dako); and CD25 (VP-C340, Vector Laboratories). The density of lymphocytic infiltrate was scored 0–5 in both the peritumoral and tumoral regions by two pathologists independently who were blinded to outcome (AA and SS).[34]

Targeted Next-Generation Sequencing

Paraffin-embedded blocks were obtained for tumor samples for 30 patients from 15 institutions across Ontario. Twenty-one samples were obtained from the primary tumor, and nine samples were from local recurrence or in-transit lesions, where the primary was not available. DNA was extracted using QIAamp DNA micro kit (QIAGEN). 25 % of

the samples, which had a delta Ct of 3.0 or had a moderate or extensive amount of melanin, underwent purification using OneStep™ PCR Inhibitor Removal Kit (Zymo Research). All samples underwent formalin-fixed, paraffin-embedded (FFPE) quality control (QC) check analysis. Twenty-six samples that passed the QC check were sequenced using Truseq Amplicon Cancer Panel on MiSeq Benchtop Sequencer (Illumina, San Diego, CA, USA). Four samples that did not pass the QC check underwent MassARRAY (Sequenom, San Diego, CA, USA) analysis. *BRAF*V600E mutation status was obtained from the clinical record of one patient.

Statistical Analysis

Response to IL-2 therapy was divided into two groups—pCR versus others. Fisher's exact test was used to determine associations between different clinicopathological variables and response to IL-2. Unpaired *t*-test was performed to determine the mean difference in time to develop in-transit disease from the diagnosis of the primary disease, and to compare the mean IHC score of ten immune markers between pCR patients versus others. Survival intervals were measured from the start of IL-2 therapy until the time of death or progression. Local progression was defined as clinical or radiological evidence of same limb cutaneous or subcutaneous lesions. Progression was defined as any type of recurrence of melanoma—local, regional (adjacent lymph-node basin), or distant. Since melanoma was a major contributing factor for all causes of death, melanoma-specific survival was not reported. Kaplan–Meier survival curves were constructed for univariate analysis for overall survival (OS), progression-free survival (PFS), and local PFS (LPFS), and log-rank *p*-values calculated. A Cox proportional hazards assumption model was used for univariate and multivariate analysis for PFS only since there were not enough observations in both groups for other endpoints. Proportional hazards assumptions for all Cox models were assessed using Schoenfeld residuals, and goodness of fit was graphically estimated using Cox–Snell residuals. Interaction analysis was performed in univariate analysis. Univariate survival analysis was also performed for CD8 and CD4:CD8 ratio. All reported *p* values were two-sided and considered statistically significant when <0.05. All statistical analysis was carried out using STATA/SE version 13.1 (StataCorp LP, College Station, TX, USA).

RESULTS

Study Population

From 2009 to 2012, a total of 32 melanoma patients were identified with in-transit disease, and were treated with intralesional IL-2. One patient was excluded from the cohort because of a concomitant diagnosis of lymphoma. The median age of the cohort at the time of initiation of IL-2 therapy was 66.5 years (range 29.1–90.5). Most of the in-transit lesions were dermal, with the minority being subcutaneous. Lesions that were injected ranged from 1 to 20 mm, and the median number of lesions injected was 16 (range 3–65). Two patients received IL-2 treatments at another institution. Details regarding patient demographics and tumor characteristics are shown in Table 1.

TABLE 1 Patient and tumor characteristics

Characteristic	*N* (%)
Primary tumor	
T status (depth, mm)	
T1 [≤ 1.0]	2 (6.5)
T2 [1.01–2]	7 (22.6)
T3 [2.01–4]	5 (16.1)
T4 [> 4.0]	15 (48.4)
Not available	2 (6.5)
Nodal status	
Negative	10 (32.3)
Positive	16 (51.6)
Not performed	5 (16.1)
Ulceration status	
Absent	11 (35.5)
Present	13 (41.9)
Not available	7 (22.6)
At start of interleukin-2 therapy	
Age (years)	
<41	2 (6.5)
41–60	4 (12.9)
61–80	17 (54.8)
>81	8 (25.8)
Sex	
Male	18 (58.1)
Female	13 (41.9)
Stage	
N2c[a]	25 (80.6)
N3[b]	2 (6.5)
IV[c]	4 (12.9)
Number of in-transit lesions	
≤16	15 (48.4)
>16	14 (45.2)
Not available	2 (6.5)
In-transit location	
Lower extremity	25 (80.6)
Upper extremity	3 (9.7)
Head and neck	3 (9.7)

[a] In-transit disease only

[b] In-transit disease with concomitant nodal metastases

[c] Distant metastasis

TABLE 2 Predictors of response to interleukin-2 therapy

Variable	*N* (%)		*p* value
	pCR	Others	
Primary tumor characteristics			
Tumor status of primary (depth, mm)			0.16
T1 (≤1.0)	2 (20.0)	0 (0)	
T2 (1.01–2)	3 (30.0)	4 (21.1)	
T3 (2.01–4)	2 (20.0)	3 (15.8)	
T4 (>4.0)	3 (30.0)	12 (63.2)	
Nodal status of primary			0.19
Negative	5 (62.5)	5 (27.8)	
Positive	3 (37.5)	13 (72.2)	
Ulceration of primary			0.39
Absent	5 (62.5)	6 (37.5)	
Present	3 (37.5)	10 (62.5)	
*BRAF*V600E mutation status			1.00
Wildtype	6 (60.0)	13 (61.9)	
Mutant	4 (40.0)	8 (38.1)	
NRAS mutation status			1.00
Wildtype	8 (80.0)	15 (75.0)	
Mutant	2 (20.0)	5 (25.0)	
Mean time from primary tumor to in-transit, months			0.44
(95 % CI)	24.8 (7.4–42.2)	44.2 (9.4–78.9)	
In-transit features at start of IL-2 therapy			
Age (years)			0.70
≤67[a]	6 (60.0)	10 (47.6)	
>67	4 (40.0)	11 (52.4)	
Sex			0.70
Female	5 (50.0)	8 (38.1)	
Male	5 (50.0)	13 (61.9)	
Stage			0.63
N2c	9 (90.0)	16 (76.2)	
N3 or IV	1 (10.0)	5 (23.8)	
Number of in-transit lesions			1.00
≤16[a]	5 (50.0)	10 (52.6)	
>16	5 (50.0)	9 (47.4)	
Location of in-transit			0.63
Lower extremity	9 (90.0)	16 (76.2)	
Upper extremity or head and neck	1 (10.0)	5 (23.8)	
Therapy-related factors			
No. of surgeries or treatments pre-IL-2 therapy			0.25
≤1	7 (70.0)	9 (42.9)	
>1	3 (30.0)	12 (57.1)	
Any systemic therapy pre-IL-2 therapy			
No	8 (80.0)	9 (42.9)	0.07
Yes	2 (20.0)	12 (57.1)	
Immunotherapy pre-IL-2 therapy[b]			
No	8 (80.0)	10 (47.6)	0.13
Yes	2 (20.0)	11 (52.4)	
Total amount of IL-2 administered (million units)			0.25
≤48.8[a]	3 (33.3)	12 (60.0)	
>48.8	6 (66.7)	8 (40.0)	
Total duration of IL-2 therapy [days]			1.00
≤75[a]	5 (50.0)	11 (52.4)	
>75	5 (50.0)	10 (47.6)	

pCR pathologic complete response, *CI* confidence interval, *IL* interleukin

[a] Cutpoints determined from median value for that variable

[b] Immunotherapy includes interferon which was administered in the adjuvant setting

Additional Treatments to IL-2 Therapy

Twenty-five patients received intralesional IL-2 therapy after failing surgical excision for in-transit disease. Six patients received IL-2 therapy at initial presentation as they had >6 lesions. Overall, either prior or post-IL-2 therapy, 18 patients received systemic therapy comprising interferon, dacarbazine, carboplatin, paclitaxel, temozolomide, vemurafenib, ipilimumab, systemic IL-2, or other investigational agents. Prior to IL-2 therapy, 14 patients received systemic therapy. After IL-2 therapy, 11 patients received repeat IL-2 treatments and six patients underwent radiation therapy for in-transit recurrence.

IL-2 Treatment Protocol

The median dose of intralesional IL-2 was 8.8 million IU/cycle (range 3.4–17.3). The median number of treatment cycles and treatment period was six cycles (range 2–13) and 2.5 months (range 0–7.2), respectively. Adverse effects were minor: one patient developed cellulitis, and most patients experienced fatigue, fever, and chills for 24 hours.

Short-Term Response to IL-2 Therapy

Post-treatment biopsies were available for 21 patients and not performed for 10 patients. Amongst the latter ten patients, biopsies were not performed for nine patients since their results would not have altered clinical management, and for one patient with a complete response who did not have a visible lesion. Overall, 10/31 (32.3 %) patients demonstrated pCR, with no evidence of disease on biopsy; 17/31 (54.8 %) patients had a partial response, and 4/31 (12.9 %) patients had progressive disease during treatment.

Genomic Analysis of Tumors

Targeted next-generation sequencing of 30 tumors identified 16 genes and 17 somatic mutations. The most frequently occurring mutations were *BRAF*V600E, 12/30 (40 %); *NRAS*, 7/30 (23 %); and *PTEN*, 2/30 (7 %). *KIT* was also identified at 1/30 (3 %).

Factors Influencing Response to IL-2 Therapy

In order to identify factors that may modulate response to IL-2 therapy, primary tumor characteristics, in-transit features at the start of IL-2 therapy, and treatment-related factors (IL-2-related, systemic, or surgical) were correlated with type of response. There were no patient factors, tumor properties or treatment factors that were associated with pCR (Table 2). Of note, six patients had nodal involvement or distant metastatic disease at the start of IL-2 therapy. Since these patients received additional treatment, the systemic impact of IL-2 therapy could not be elicited on nodal or distant metastases.

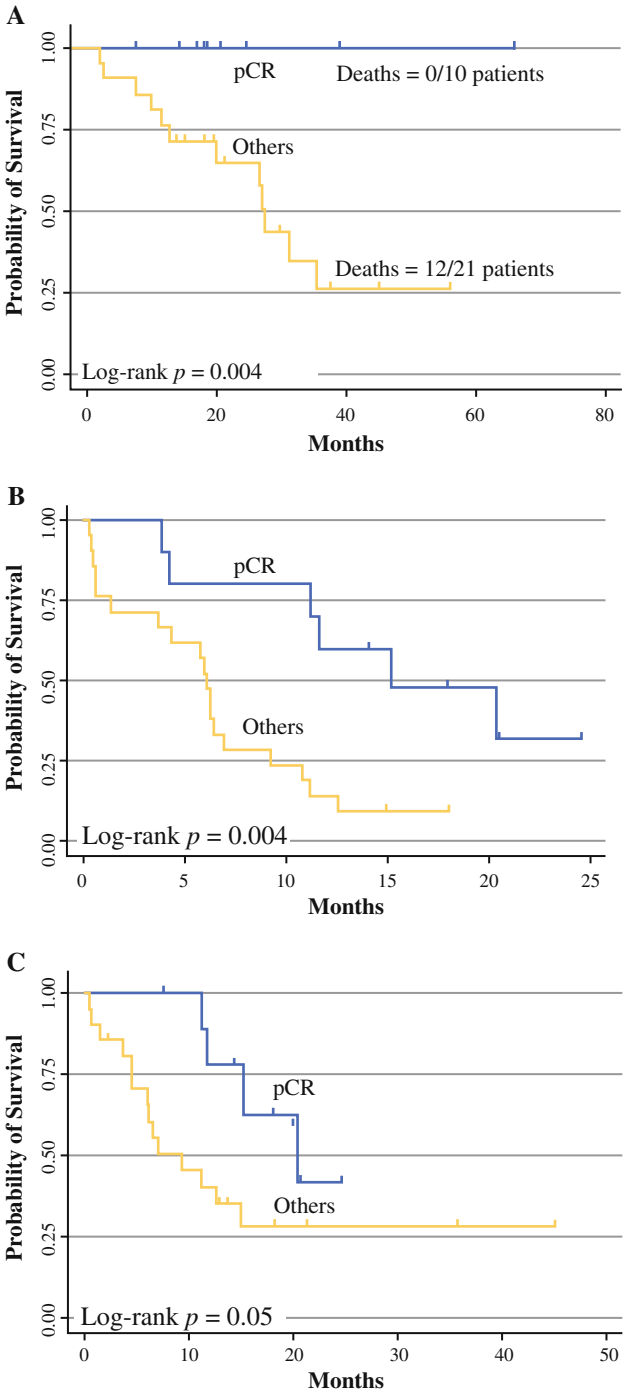

FIG. 1 Unadjusted Kaplan–Meier survival curves comparing patients with pCR versus others in **a** overall survival, **b** progression-free survival, and **c** local progression-free survival. *pCR* pathologic complete response

Long-Term Response to IL-2 Therapy

The median follow-up from the start of IL-2 therapy was 19.4 months (range 2.0–65.8 months). After treatment with intralesional IL-2 therapy, 18/31 (58.1 %) developed local progression and 25/31 (80.6 %) patients developed progression (local, regional, or distant). Twelve patients (12/31, 38.7 %) died, and melanoma was the major contributing cause of death for all patients. Of the ten patients who developed pCR, four locally progressed, six progressed, and none of the patients died.

pCR to IL-2 therapy was associated with OS (log-rank $p = 0.004$), PFS, (log-rank $p = 0.004$), and LPFS (log-rank $p = 0.051$) [Fig. 1]. Univariate analysis for PFS was performed for tumor-related[6] and treatment-related variables. Variables with strong clinical rationale or statistical significance in univariate analysis were selected for multivariate analysis ($n = 29$). Patients with pCR to IL-2 therapy demonstrated an improved PFS after multivariate analysis (adjusted hazard ratio [HR] 0.11; 95 % confidence interval [CI] 0.02–0.47; $p = 0.003$) [Table 3]. No interaction was identified between IL-2 response and advanced stage ($p = 0.70$), amount of IL-2 injected ($p = 0.54$), duration of treatment ($p = 0.67$), or systemic therapy ($p = 0.52$). Therefore, the impact of pCR upon PFS is independent of the stage at the start of IL-2 treatment, amount of IL-2 injected, duration of IL-2 treatment, and systemic therapy.

Mechanism of Intralesional IL-2 Therapy

IHC from a panel of ten immune markers revealed low intratumoral variability (Fig. 2a), and therefore the mean peritumoral IHC score was compared between those patients with a pCR ($n = 9$) and others ($n = 9$) [data not shown]. The mean IHC score for the peritumoral CD8+ infiltrate was higher in patients with pCR versus others (3.78 vs. 2.61; $p = 0.01$) [Fig. 2b, c], and the CD4:CD8 ratio was lower in patients with pCR compared with others (0.65 vs. 1.04; $p = 0.01$). Patients with an elevated CD8+ infiltrate (IHC score >2) demonstrated an improved PFS (unadjusted HR 0.08; 95 % CI 0.01–0.52; $p = 0.008$), which was consistent when CD8+ score was analyzed as a continuous variable (unadjusted HR 0.47; 95 % CI 0.23–0.99; $p = 0.046$). An elevated ratio of CD4:CD8 T-cell infiltrate was associated with poorer PFS (unadjusted HR 4.02; 95 % CI 1.16–13.92; $p = 0.03$).

DISCUSSION

Although several studies have reported the use of intralesional IL-2 for in-transit disease, there is a broad range of short-term response rates, from 25 to 96 %.[21,24–27,31] This may be due to different dosing regimens and intervals, and varying definitions of response. The methodology used in our study is similar to what was employed by Boyd et al.[24,33] with biweekly injections of IL-2 and biopsies

TABLE 3 Unadjusted and adjusted progression-free survival analysis

	Unadjusted		Adjusted	
	HR (95 % CI)	*p* value	HR (95 % CI)	*p* value
Response to IL-2				
pCR vs. others	0.26 (0.09–0.71)	0.009	0.11 (0.02–0.47)	0.003
Stage at start of IL-2 therapy				
N3/IV vs. N2c	2.04 (0.78–5.30)	0.15	5.94 (1.58–22.3)	0.008
Total quantity of IL-2 given				
Hi vs. Lo[a]	0.41 (0.17–0.95)	0.04	0.52 (0.19–1.39)	0.19
Duration of IL-2 treatment				
Hi vs. Lo[a]	0.35 (0.16–0.79)	0.01	0.10 (0.03–0.36)	<0.001
Systemic therapy (pre-/post-IL-2)				
Yes vs. no	2.49 (1.05–5.92)	0.04	0.30 (0.08–1.11)	0.07
Primary tumor ulceration				
Presence vs. absence	1.34 (0.56–3.19)	0.51	–	–
Intralesional IL-2 for recurrence[b]				
Yes vs. no	1.36 (0.60–3.07)	0.46	–	–
Radiation for recurrence[b]				
Yes vs. no	1.11 (0.44–2.79)	0.83	–	–

HR hazard ratio, *CI* confidence interval, *IL* interleukin, *pCR* pathologic complete response

[a] Hi vs Lo refers to two groups with the median value used as a cutpoint

[b] Recurrence refers to in-transit recurrence post-IL-2 therapy

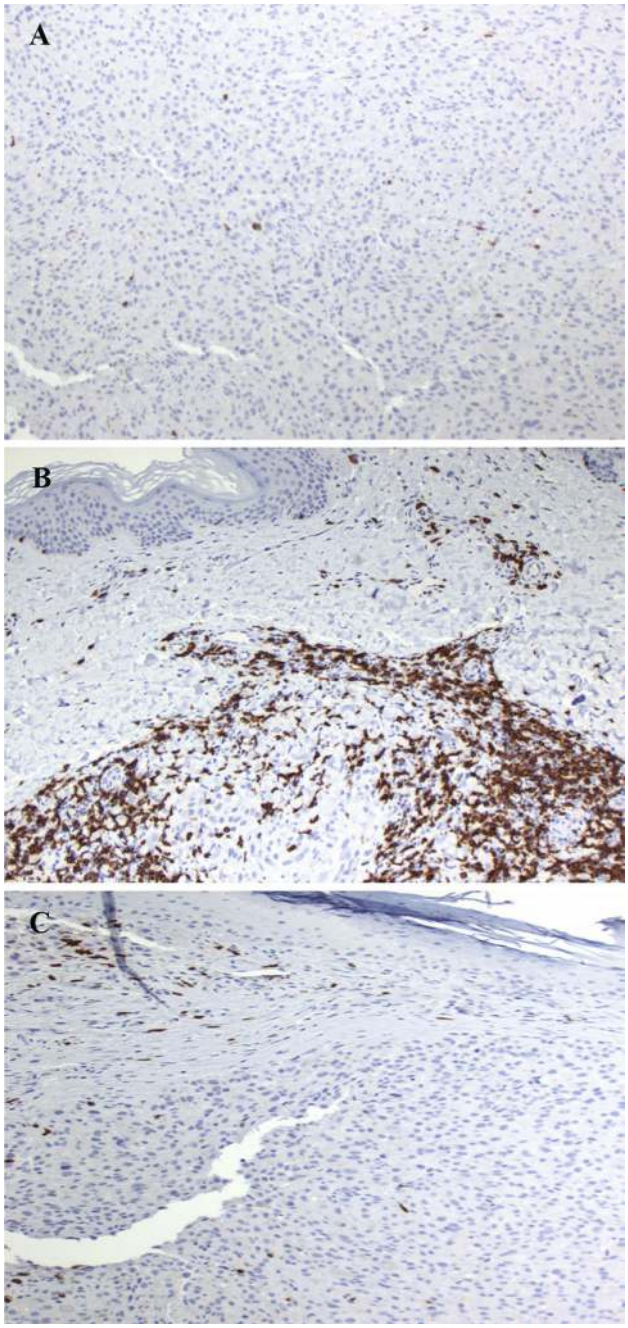

FIG. 2 Immunohistochemical analysis of in-transit lesions post-intralesional interleukin-2 therapy using 10x objective lens magnification. **a** Low intratumoral CD8+ infiltrate; **b** high peritumoral CD8+ infiltrate; **c** low peritumoral CD8+ infiltrate

performed to document response to treatment. We demonstrated a pCR rate of 32 % and a combined response (complete and mixed) rate of 87 %. Boyd et al. reported a pCR rate of 51 % and a combined response rate of 82 %.[24] These results are in a similar range and the small variability is plausible because of the small sample size in our study ($n = 31$) and that of Boyd et al. ($n = 39$).[24] Therefore, our results are comparable to what was previously reported.

This is the first study to report an association of pCR to PFS in multivariate analysis. No interaction was found with tumor-related and treatment-related factors. Only two patients who achieved pCR also received systemic therapy prior to or post IL-2 therapy (data not shown), and there was no interaction between response to IL-2 therapy and systemic therapy, suggesting that systemic therapy was not a contributing factor to the improved survival for pCR patients. Therefore, due to the low morbidity of intralesional IL-2 injections, this therapeutic strategy should be offered as a first line treatment once surgical resection is no longer feasible for in-transit metastases.

We found that the frequency of mutations was similar to what was previously reported for in-transit and melanoma patients overall: 40 %, *BRAF*V600E; 21 %, *NRAS*; and 3 %, *KIT*.[35–37] Although we did not find that *BRAF*V600E or *NRAS* mutations influenced response to intralesional IL-2 therapy, this may be due to our smaller cohort size. *NRAS* mutation status was previously shown to be associated with response to intravenous IL-2 therapy in a larger cohort of patients with metastatic melanoma.[38]

In order to better understand the mechanism of IL-2 response, we studied a panel of immune markers from post-treatment biopsies. Similar to previously reported studies, a peritumoral T-cell-rich infiltrate was identified.[26,32] There are other immunotherapies, such as ipilimumab or anti-PD-1 (programmed death-1) therapy, that also have a T-cell-mediated response. Furthermore, BRAF inhibitors can modulate the tumor microenvironment by enhancing the CD8+ T-cell infiltrate, suggesting the potential for combining BRAF inhibitors or immunotherapies with localized IL-2 therapy.[39–43]

We found that patients who achieved pCR had a more pronounced CD8 + T-cell infiltrate, which was associated with an improved PFS. This is suggestive that there may be a systemic immune effect from localized IL-2 treatment. Interestingly, local administration of IL-2 was previously shown to induce a systemic response in patients.[44] Intratumoral injections of IL-2 also demonstrated a systemic impact upon contralateral tumors in vivo.[45] Localized IL-2 therapy increased IL-2 concentrations in the tumor microenvironment that stimulate T and natural killer (NK) cells, which diffuse in extracellular fluid into the circulation, and which may help explain the systemic response.[46] Furthermore, the concept of a systemic impact from localized treatment has been observed with other intralesional agents such as PV-10, Allovectin-7, and Oncovex$^{GM\text{-}CSF}$/T-Vec.[47,48]

CONCLUSIONS

Intralesional IL-2 therapy is an effective treatment modality for melanoma patients with in-transit disease with low morbidity. Importantly, our study has illustrated a

strong and independent association between pCR to IL-2 therapy and PFS, suggesting that intralesional IL-2 can improve the long-term survival for a cohort of patients with in-transit disease. Our results point the way for further investigations in larger patient cohorts and potential combination therapies.

ACKNOWLEDGMENT The authors would like to thank the institutions across Ontario who helped provide paraffin-embedded blocks for this study, including University Health Network, Barrie Royal Victoria Hospital, Muskoka Algonquin Healthcare, Headwaters Health, Orillia Soldier's Memorial Hospital, West Parry Sound Health Centre, Lifelabs, Gamma Dynacare, Windsor Regional Hospital, Ross Memorial Hospital, Peterborough Regional Health Centre, London Health Sciences Centre, St. Mary's General Hospital, Kitchener, Kingston General Hospital, and Grand River Hospital. This study was supported by the William S. Fenwick Fellowship and the Joseph M. West Family Memorial Fund from the 2012 Postgraduate Medical Research Award, awarded to Saima Hassan, University of Toronto; Melanoma Site Group from Sunnybrook Health Sciences Centre, awarded to Teresa Petrella; and a Research Grant from Pathology Associates from the University Health Network, awarded to Danny Ghazarian.

DISCLOSURE Novartis provided interleukin-2 to patients free of charge as part of a compassionate release program. Novartis was not involved in funding this research study. Teresa Petrella and an immediate family member received honoraria from Novartis, Roche, BMS, Merck, GSK, Amgen, AstraZeneca, Electa, Janssen, Paladin, and Sanofi; they served as a consultant or in an advisory role for Novartis, Roche, BMS, Merck, GSK, Amgen, Astellas, Janssen, and Sanofi. An immediate family member of Teresa Petrella was paid to speak at a speaker's bureau from Amgen, and has received research funding from Sanofi and Paladin. The University Health Network, Toronto, ON, Canada, where Suzanne Kamel-Reid is an employee, receives research funding from Novartis. Saima Hassan, Tong Zhang, Francesco Nordio, Andrea Baccarelli, Shachar Sade, Karen Naert, Ayman Al Habeeb, Danny Ghazarian, and Frances Wright have nothing to disclose.

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