Egyptian Journal of Medical Microbiology Volume 28 / No.3 / July 2019 149-154 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Subcutaneous Versus Intranodal Dendritic Cells- DRibbles Vaccination of Hepatocellular Carcinoma in Animal Model

Awny A. Gawish, Takwa E. Meawed, Alia A. El Shahaway, Naglaa M. Ahmed*
Department Medical of Microbiology& Immunology, Zagazig University, Egypt

Key words:
Autophagy; dendritic cells; hepatocellular carcinoma; antitumor immune response

Corresponding Author:
Naglaa Mohamed Mohamed Ahmed
Department Medical of Microbiology& Immunology, Zagazig University, Egypt
Tel.: 01149174062
nag_kasas@yahoo.com

ABSTRACT

Background: *Cancer immunotherapy became a promising alternative to old therapeutic options in hepatocellular carcinoma. DC-DRibbles vaccine has clinical applications across a broad range of malignancies.* ***Objective:*** *To compare the efficacy of subcutaneous and intranodal routes of injection of Dendritic Cells-DRibbles immunotherapy in HCC induced mice.* ***Methodology:*** *This experimental study was conducted on 24 BALB/c mice. Healthy as negative control and cancer induced. The later was subdivided into two groups: positive control group and vaccinated groups (A and B) according to route of administration.* ***Results:*** *The mean of percentage of tumor volume reduction was 92.76% in DC+ Dribbles intranodal group (B), which was better than mean of percentage of change in subcutaneous group (A) which was 90.87%.* ***Conclusion:*** *DC- Dribbles vaccine was effective as HCC immunotherapy, both subcutaneous and intra nodal routes had comparable results. This study recommends subcutaneous route over intranodal route immunotherapy; it is simple, less invasive and effective.*

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the sixth most common form of cancer worldwide with delayed diagnosis few therapeutic options [1,2]. Immunotherapy by using immune checkpoint inhibitors and Several antibodies targeting specific tumor-associated antigens (TAAs) have also been proposed as immunotherapy approach for HCC treatment [3,4].

Dendritic cells are professional APCs, capture antigens through several complementary mechanisms. Antigen-loaded DCs migrate into the draining lymph nodes. Meanwhile, they process the proteins into peptides that bind to both MHC class I molecules and MHC class II molecule[5]. Immunotherapy strategy that can be applicable for HCC treatment is the vaccination based on TAAs with different targets and methodological approaches are under investigation in several clinical trials[6].

The essential step for the activation of the immune response against tumor cells is the priming of naïve T cells, able to recognize specific tumor antigens on surface of antigen presenting cells (APCs). One way to achieve this activation is through vaccination that, as endpoint, may provide an inhibition of advanced or refractory tumor growth[7].

Induction of autophagy in Hep-G2 cells and isolation of mature autophagosome which can be considered bag full of tumor antigens sourses, these vesicles are termed Dribbles[8]. DRibbles used as a potent antigen source in cross-presentation assays and in in vivo vaccine studies[9]. Investigators observed that vaccination with antigens derived from autophagosomes can broaden the T-cell protective immune response[10].

Thus, there appears to be some controversy in the literature regarding the optimal route of immunization with a DC based vaccine, and these routes of immunization have not reached a satisfactory level[11]. In this work, we compared efficacy of the subcutaneous and intranodal route of injection of DC-DRibbles immunotherapy in HCC induced mice.

METHEDOLOGY

Study design and subjects

This experimental study was conducted at the Immunology Research Laboratory, Microbiology and Immunology Department, Animal Care Center, Faculty of Medicine, Zagazig University, Egypt from May 2018 to February 2019.

This study included 24 BALB/c mice (female 6–8 weeks of age), Weight (19-22 gram). Mice were purchased from VACCERA Animal Care Center Dokki, Egypt and maintained under specific pathogen-free conditions.

Healthy (negative control) (6 mice) and cancer induced (18 mice). Cancer induced group was subdivided into two groups: Untreated positive control group (6 mice) and group treated with Dendritic Cells-DRibbles (12 mice), that was further subdivided into 2 subgroups according to route of injection: group (A) injected subcutaneous (6 mice) Group (B) injected intranodal (6 mice).

Mice were maintained under optimal light, temperature, humidity and specific pathogen-free condition. Mice were sacrificed when signs of suffering were observed such as reduced mobility and altered behavior in accordance with the protocol approved by the Institutional Animal Care and Use Committee. Zagazig University (IACUCC) Number: ZU-IACUCC/3/F/54.

Methods:

Subcutaneous injection of HepG2 **tumor cells in humanized HCC mouse model:**

HepG2 epithelial cell line was purchased from Nile Center for Experimental Researches (NCER), Mansoura**.** Cancer cell line was cultured in DMEM (Gibco) supplemented with 10% FBS (Invitrogen), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco) at 37°C, 5% CO_2 incubator see figure 1. Eighteen female BALB/c mice were injected with 2.5×10^6 humanized HepG2 cells subcutaneously in the lower right flank.

Monocytes- derived DCs:

Fresh peripheral blood was obtained from healthy donors. Heparinized blood was separated by density gradient (Ficoll-Hypaque), every two days the cells were fed with fresh RPMI medium containing GM-CSF (R&D Systems, catalog no 215-GM) and IL-4 (R&D Systems, catalog no 204-IL) **.** At day six the maturation was done by Resiquimod (R848) (TOCRIS, Catalog no. 4536) for additional two days.

Preparation of Dribbles vaccine

HepG2 cells were cultured in DMEM complete medium containing 10% FBS and treated with rapamycin (autophagy inducer) and chloroquine (inhibit autophagosome lysosome fusion) for 24 h in CO_2 incubator. The resulting suspension was pre-cleared by differentrial centrifugation at 1600 r/min for 10 min then 30-min centrifugation at 12,000 r/min followed by washing with (PBS) and recentrifuged again for 30 min at 12,000 r/min. The total protein concentration was measured by bicinchoninic acid (BCA) assay according to the manufacturer's protocol (Thermo catalog no 23227). Autophagosomes formation were detected by by Flow Cytometry using Cyto ID stain (Autophagy Detection Kit ab139484 (Enzo Life Sciences)

Dribbles-loading onto DCs

The mature DCs were loaded with (Dribbles) from Hep-G2 cancer cell line. They are co-cultured in 6-well plates for an additional 24 h at 37°C, 5% CO_2. Expression of CD80, CD86 and HLA-DR on the surface of Mature DC before and after coculture with Dribbles was detected by flow cytometry.

The schedule of vaccine injection:

On day 14 after HCC induction in mice, average volume of tumors had reached about 80 mm^3, tumor-bearing mice were divided randomly into three groups: Positive control group (6mice) were treated with PBS(Sigma) subcutaneously two doses with one week apart. Group (A) were treated with HepG2-derived Dribbles plus DC two doses one week apart subcutaneously injected into both flanks. Group (B) were treated with HepG2-derived Dribbles plus DC, two doses one week apart injected into both inguinal lymph nodes.

Subcutaneous vaccine injection

The vaccine 100ul (1×106 DCs and 200ug DRibbles), was loaded into an insulin syringe with a 25 Gauge needle. The needle was inserted into the subcutaneous space. Leakage out at puncture site can be avoided by advancing the needle several millimeter through the space before needle withdrawal [12, 20].

Intranodal vaccine injection (Inguinal lymph node)

Anesthesia of mice: (Ketamin + Xylazine) given intraperitoneally by 25G needle.

Operation: The mice were put on its back, the hair was removed, the inguinal region was disinfected with ethanol 70%, the hip joint was bent 90º, curved microdissecting forceps were used to hold the skin up, small incision was cut using surgical incissors, the incision was widened by opening the incissors, light guide was used to improve visibility. Ten ul of vaccine (1×10^6 DCs and 30µg DRibbles) was aspirated, the syringe was free of air, the lymph node was localized with curved forceps and tip of closed cissor. The L.N appear greyish within the more whitish fat tissue, afferent and efferent capillaries were seen entering and leaving the L.N, the needle was inserted into the node with bevel facing up. The incision was closed by suturing.

Post operation*:* The mice were put in cage and kept warm, the mice were observed until they wake up, the wound typically healed within seven days[13]**.**

Tumor volume measurement

Tumor volume was calculated according to formula: Volume = Length × $(Width)^2$ /2. Until day 31 Mice were sacrificed by (Halothane inhalation overdose) when the tumor volume had recorded 200 mm^2 or larger.

Statistical analysis

The collected data were analyzed by SPSS 24 (SPSS Inc., Chicago, IL). Mann-Whitney U (MW test) was used for Comparing numerical variables between 2 groups Kruskal Wallis test (KW test) was used for comparing numerical variables between more than 2 groups. The results were considered statistically significant when the significant probability was less than 0.05 ($P < 0.05$).

RESULTS

Detection of autophagosome by flow cytometry:

The results of flow cytometry-based analysis of Hep-G2 cell, control cells showed low fluorescence signal intensity. In the samples treated with Rapamycin and chloroquine for 24 hours, the Green Detection

Reagent (Cyto-ID) signal increased, indicating an increase in autophagic vesicles with LC3II in Hep G2 cells as shown in (Figure 1).

Tumor volume:

At the end of the study, the mean of tumor volume was 8.5 ±4.44 in group (A) DC+ Dribbles subcutaneous vaccine, while it was 7.41 ± 3.35 in group (B) DC+ Dribbles intra nodal vaccine with no significant difference (p=0.785). In positive control group (treated with PBS), the mean of tumor volume was 213.33±10.32 mm^3 with highly significant difference with treated groups. As shown in (Table 1).

There is no statistically significant difference in the mean of percentage of tumor volume reduction in group A (90.87±15.68) and B (92.76±2.91) (P= 0.873) as shown in (Table 2).Unfortunately during intranodal injection, one mice in group C2 died on table in operation with failure of resuscitation, so the number of mice in group B was reduced to 5 instead of 6 mice.

Table (1): Volume of tumor at the end of the study among the different groups.

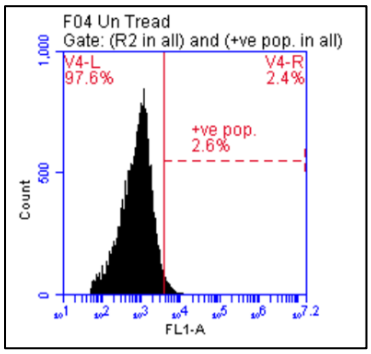
Volume of tumor	Control Group(PBS) (N=6)	Group A DC+ Dribbles (subcutaneous) (N=6)	Group B DC+ Dribbles (Intranodal) (N=5)
Mean ± SD	213.33±10.32	8.5 ±4.44	7.41 ± 3.35
Median	220	8	8.2
Min-max	200-220	2-13.5	2-11
#P- value of Kruskal Wallis test: 0.000 (HS)			
P-value of Mw test ‡	Reference	0.000(HS)	0.000(HS)
		Reference	0.785 (NS)

Table (2): The percentage of tumor volume reduction between two groups.

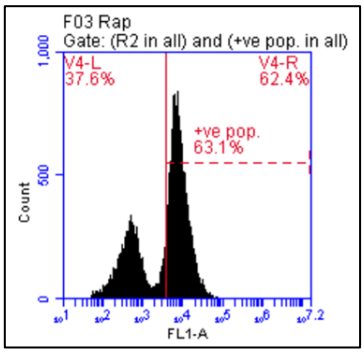
Percentage of change (%)	Group A DC+ Dribbles (subcutaneous) (N=6)	Group B DC+ Dribbles (Intranodal) (N=5)
Mean ± SD	90.87±15.68	92.76±2.91
Median	92.2	92.1
Min-max	97.7- 82	97.33- 89.5
P-value of Mw test ‡	Reference	0.873 (NS)

‡ Mann-Whitney for comparison between 2 groups

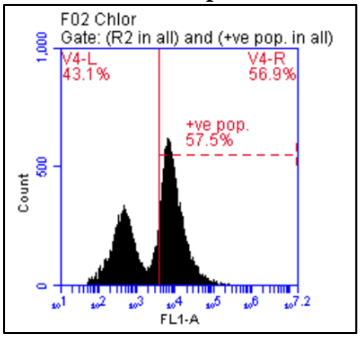
Significant P-value <0.05 ***NS: non-significant*** ***S: Significant***



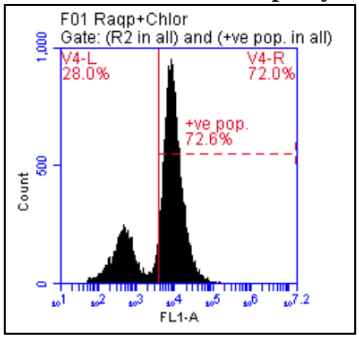
Untreated HepG2 cells



Cells treated with rapamycin



Cells treated with chloroquine



Cells treated with rapamycin and chloroquine

Fig (1): Detection of autophagosome by flow cytometry.

DISCUSSION

The introduction of immunotherapy in the context of HCC has rapidly increased the treatment options and response rates of HCC patients[14]. Initial promising results of new immune therapies as dendritic cell based vaccination, combining with locoregional treatments or surgery used for the treatment of HCC patients[15, 16].

This experimental study was designed to compare the efficacy of the subcutaneous and intranodal route of injection of the DC-DRibbles immunotherapy in HCC induced mice. Our work included randomly collected 24 mice, Healthy (negative control) (6 mice) and cancer induced (18 mice). Cancer induced group was subdivided into two groups: Untreated positive control group (6 mice).Treated group (12 mice), that was subdivided into: Group (A) was treated with DC-Dribbles subcutaneously (6 mice), group (B) was treated with DC- Dribbles into inguinal lymph nodes (5 mice).

The DRibble-DCs vaccine could effectively activate T cells, which secreted the cytokine IFN-γ and inhibited tumor growth. Furthermore, immunization with DRibble-DCs could reduce the proliferation of T-regulatory cells to relieve tumor immunosuppression[17,18].

Current immunotherapy schedule was started two weeks after subcutaneous induction of Hep-G2 cancer cell line in mice, with average tumor volume (80-100) mm^3 in all studied groups. Our findings showed that Dribbles- pulsed DC immunotherapy had great effect on HCC tumor regression compared to control group. This was in accordance with different studies on multiple cancer cell lines [9,17,19, 20]. At the end of the study, the mean of tumor volume in group B (intra nodal) was less than that in group A (subcutaneous) with no statistically significant difference between them (Table 1). The results of DC-Dribbles intranodal vaccine were just superior to DC-DRiblles subcutaneous vaccine results and they were comparable at the end of the study at 31th day, with no significant differences (p=0.873).

In current work we compared between two routes of vaccine injection, the mean of percentage of tumor volume reduction was 92.76% ± 2.9 in DC+ Dribbles intranodal group (B), which was better than mean of percentage of change in subcutaneous group (A) which was 90.87% ± 15.68 (table 2). However, there was no statistically significant difference in percentage of tumor volume reduction between both groups (P=0.873). In this study, the risk of complications (death) during intranodal injection was =17%.

More than twenty years ago, this technique (intranodal injection of anticancer drugs) had been tried by Osaki et al[21]. Sato and his colleagues[22] found that intralymphatic chemotherapy in mice in lymph nodes has marked anti-tumor effect. Also, Johansen and his colleagues[23] study was in agreement with our results, they compared three routes of peptide vaccine to lymphoma in C57BL/6 mice, intravenously, subcutaneously and directly into lymph node, they confirmed that the intra nodal administration route was superior to other routes with respect to the frequency of IFN-α producing CD8 T cells with a CD44 high memory phenotype.

However, this result was contrary to Lesterhuis et al study[24] the intranodal route of administration did not offer an advantage over intradermal vaccination beside, it was more laborious and variable. The intra-lymphatic method is more invasive than other injectable methods such as IV and SC injections, and the technique of injection is difficult; an improper injection could disrupt the lymph node architecture.

Bedrosian and his colleagues[25] approved that intranodal DC vaccination resulted in professional T cell immune reaction compared to intradermal vaccination. Verdijk et al[26] observed that higher percentages of DCs migrate to lymph nodes after intranodal vaccination when injected correctly, while low percentage reach subsequent lymph nodes after subcutaneous injection, immune responses were included in both routes of vaccination despite these differences. Induction of antigen-specific immune responses need limited numbers of DC in the draining lymph nodes.

After more than one hundred intranodal injections, Adamina and his colleagues[27] did not observe any complications related to the application of this technique in their clinical study in melanoma patients.

Limitations of intra lymphatic immunization include, the efficiency of this method is more operator dependent than other immunization methods, localization of lymph node is difficult in small mice less than 5 weeks and experience is needed[13]. For weakly immunogenic antigens, Intranodal immunization is preferable to conventional methods such as subcutaneous or intramuscular routes[28].

We recommend subcutaneous route of vaccination, it is simple, effective and less invasive technique. Advances in intra vital microscopy, mainly in mice studies have provided a window on the migratory behavior and interactions of DCs and T cells inside the lymphoid microenvironment. The time needed by activated DC to reach the draining LN is 24-72 hours after stimulation [29].

The skin offers a rich immune network comprised of Langerhans cells in the epidermal compartment and dermal DCs. Local APC are accompanied by specialized cells with immune function, including macrophages, keratinocytes, mast cells, natural killer (NK) T cells, and fibroblasts, with access to draining lymphatic and blood vessels. These characters make the skin an ideal route for DC-based vaccination [28, 29].

CONCLUSION

DC- Dribbles vaccine was effective antitumor immune response in HCC treatment. DC+ Dribbles subcutaneous vaccine had comparable results with DC+ Dribbles intranodal vaccine in tumor volume reduction with no statistically significant differences.

Recommendation:

We recommend DC- Dribbles subcutaneous route of vaccination in HCC treatment, it is simple, effective and less invasive technique.

Conflicts of interest:

The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

REFERENCES

1. Busato D, Mossenta M, Baboci L, Di Cintio F, et al. Novel immunotherapeutic approaches for hepatocellular carcinoma treatment; Expert Review of Clinical Pharmacology. DOI: 10.1080/17512433.2019.1598859
2. Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, et al .Hepatocellular carcinoma. Nat Rev Dis Primer. 2016; 2:16018.
3. Daher S, Massarwa M, Benson AA, Khoury T. Current and Future Treatment of Hepatocellular Carcinoma: An Updated Comprehensive Review. J Clin Transl Hepatol. 2018; 6:69–78.
4. Yong CSM, Dardalhon V, Devaud C, Taylor N, et al. CAR T-cell therapy of solid tumors. Immunol Cell Biol. 2017; 95:356–63.
5. Anguille S, Smits EL, Lion E, van Tendeloo VF, Berneman ZN. Clinical use of dendritic cells for cancer therapy. Lancet Oncol. 2014; 15: 257–267.
6. Buonaguro L, Mayer-Mokler A, Accolla R, Ma Y T, Hiedenreich R, Avallone A et al. HepaVac-101 first-in-man therapeutic cancer vaccine phase I/II clinical trial for hepatocellular carcinoma patients. J of clin oncol. 2018; 36(15 suppl) p. TPS3135.
7. Hu Z, Ott PA, Wu CJ. Towards personalized, tumour-specific, therapeutic vaccines for cancer. Nat Rev Immunol. 2017; 18:168–82.
8. New J, Thomas S M. Autophagy-Dependent Secretion: Mechanism, Factors Secreted, and Disease Implications, Autophagy, DOI:10.1080/15548627.2019.1596479
9. Twitty CG, Jensen SM, Hu HM, Fox BA. Tumor derived autophagosome vaccine: induction of cross protective immune responses against short-lived proteins through a p62-dependent mechanism. Clin Cancer Res. 2011; 17:6467-81.
10. Zhou M, Wen Z, Cheng F, Ma J, Li W, et al. Tumor-released autophagosomes induce IL-10-producing B cells with suppressive activity on T lymphocytes via TLR2-MyD88-NF-kB signal pathway. Oncoimmunology. 2016; 5(7): e1180485.
11. Shang N, Figini M, Shangguan J, Wang B, et al. Dendritic cells based immunotherapy. Am J Cancer Res.2017; 7: 2091–2102.
12. Reuter J. Sub cutaneous injection of tumour cells. Bioprotocol Bio 101:e166.DOI:10.2179 /Bioprotec.166.2011
13. Johansen P, Kunding TM. Intralymphatic immunotherapy and vaccination in mice Article in Journal of Visualized Experiments.2014; (84) doi: 10.3791/51031.
14. Fan Y, Shu L, Ding X, Yue J, Jiang J, et al. First-in-class immune-modulating small molecule I caritin in advanced hepatocellular carcinoma: preliminary results of safety, durable survival and immune biomarkers. 2019; BMC Cancer 19: 279 2 - 11
15. Lurje I , Czigany Z , Bednarsch J, Roderburg C, et al. Treatment Strategies for Hepatocellular Carcinoma—A Multidisciplinary Approach Int. J. Mol. Sci. 2019; 20(6)1465.
16. Longo V, Gnoni A, Gardini AC, Pisconti S, et al. Immunotherapeutic approaches for hepatocelleular carcinoma oncotarget. Journals Advance Publications. 2017; 8 (20): 33897-33910.
17. Dong H, Su H, Chen L, Liu K, Hu H, et al. Immunocompetence and mechanism of the DRibble-DCs vaccine for oral squamous cell carcinoma. Cancer Management and Research. 2018; 10: 493–501.
18. Page DB, Hulett TW, Hilton TL, Hu HM, et al. Glimpse into the future: harnessing autophagy to promote anti-tumor immunity with the DRibbles vaccine. Journal for ImmunoTherapy of Cancer. 2016; 4: 25.
19. Li Y, Wang LX, Pang P, Cui Z, Aung S, et al. Tumor-derived autophagosome vaccine: mechanism of cross-presentation and therapeutic efficacy. Clin Cancer Res.2011; 17: 7047-57.
20. Su S, Zhou H, Xue M, Liu J, Ding L, et al. Anti-tumor Efficacy of a Hepatocellular Carcinoma Vaccine Based on Dendritic Cells Combined with

Tumor-derived Autophagosomes in Murine Models Asian Pacific Journal of Cancer Prevention. 2013; 14 (5): 3109-3116.

21. Osaki T, Ueta E, Yoneda K, Yamamoto T. Intranodal injection of anticancer drugs into fixed cervical metastatic lymph nodes. Oral Dis.1997; 3(4):247-53.
22. Sato T, Mori S, Arai Y, Kodama T. The combination of intralymphatic chemotherapy with ultrasound and nano-/microbubbles is efficient in the treatment of experimental tumors in mouse lymph nodes. Ultrasound Med Biol. 2014; 40(6):1237-49.
23. Johansen P, Haffner A C, Koch F, Zepter K, et al. Direct intralymphatic injection of peptide vaccines enhances immunogenicity; Eur. J. Immunol. 2005; 35: 568–574.
24. Lesterhuis W, De Vries I, Schreibelt G, Lambeck AJ, Aarntzen EH, et al. Route of administration modulates the induction of dendritic cell vaccine-induced antigen-specific T cells in advanced melanoma patients. Clin Cancer Res. 2011; 17(17): 5725-35.
25. Bedrosian I, Mick R, Xu SW, Nisenbaum H, et al. Intranodal administration of peptide-pulsed mature dendritic cell vaccines results in superior CD8+ T-cell function in melanoma patients. Journal of Clinical Oncology. 2003; 21(20):3826-35.
26. Verdijk P, Aarntzen E H, Lesterhuis W J, Boullart A C, et al. Limited Amounts of Dendritic Cells Migrate into the T-Cell Area of Lymph Nodes but Have High Immune Activating Potential in Melanoma Patients Clin Cancer Res.2009 ;15(7) 2531-2540.
27. Adamina M , Rosenthal R, Weber W P, Frey D M, et al. Intranodal Immunization With a Vaccinia Virus Encoding Multiple Antigenic Epitopes and Costimulatory Molecules in Metastatic Melanom. Molecular Therapy. 2010; 3(18)651-659. http://doi.org/ 10.1048/mt.2009.275.
28. Fehres CM, Garcia-Vallejo JJ, Unger WW, van Kooyk Y (2013): Skin-resident antigen- presenting cells: instruction manual for vaccine development; Front Immunol (4)157.
29. Pizzurro GA, Barrio MM (2015): Dendritic cell-based vaccine efficacy: aiming for hotspots; Frontiers in Immunology (6) 91.