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ORIGINAL ARTICLE

Intratumoral gene therapy versus intravenous gene therapy for distant metastasis control with 2-Diethylaminoethyl-Dextran Methyl Methacrylate Copolymer Non-Viral Vector–p53

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Lung cancer still remains to be challenged by novel treatment modalities. Novel locally targeted routes of administration are a methodology to enhance treatment and reduce side effects. Intratumoral gene therapy is a method for local treatment and could be used either in early-stage lung cancer before surgery or at advanced stages as palliative care. Novel non-viral vectors are also in demand for efficient gene transfection to target local cancer tissue and at the same time protect the normal tissue. In the current study, C57BL/6 mice were divided into three groups: (a) control, (b) intravenous and (c) intatumoral gene therapy. The novel 2-Diethylaminoethyl-Dextran Methyl Methacrylate Copolymer Non-Viral Vector (Ryujyu Science Corporation) was conjugated with plasmid pSicop53 from the company Addgene for the first time. The aim of the study was to evaluate the safety and efficacy of targeted gene therapy in a Lewis lung cancer model. Indeed, although the pharmacokinetics of the different administration modalities differs, the intratumoral administration presented increased survival and decreased distant metastasis. Intratumoral gene therapy could be considered as an efficient local therapy for lung cancer.

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Keywords: vectors; intratumoral; intravenous; lung cancer; p53; DDMC

INTRODUCTION

Lung cancer treatment in an evolving field as novel pathways and gene mutations are being discovered.[1] Until recently, non-specific cytotoxic drugs were administered as first-line treatment; however, with the evolving science of pharcogenomics, agents targeting the mutations of lung cancer were introduced in the market as first-line treatment.[2–4] Several new pathways are being investigated as possible targets for inhibition, and lung cancer treatment is directed to being personalized.[5–7] Administering non-specific cytotoxic agents by intravenous route or oral targeting agents has, for many patients, adverse effects that can potentially postpone their treatment.[8–10] Therefore the concept of delivering the necessary dose of treatment directly to the target tissue has been investigated with (a) gene therapy, (b) immunotherapy and (c) chemotherapy or combinations of the above methods.[11–17] The following methods for intratumoral treatment have been used: (a) brachytherapy, (b) photodynamic therapy, (c) thermal and non-thermal ablative therapies, (d) chemotherapy, and (e) gene therapy.[16,18–20] Gene therapy has been used for lung cancer to sensitize cells to radiotherapy and chemotherapy.[21–23] Gene therapy is used to insert genetic material into a cell. There are currently two vehicles that are used for efficient gene transportation: the viral and the non-viral vectors. There are advantages and disadvantages for each vehicle. The viral vectors tend to induce neutralizing antibodies known as NABs within 3–7 days, and several non-viral vectors have a low DNA uptake capability and have been observed to be toxic for certain normal cells, such as the airway epithelium.[24–28] The intratumoral treatment efficiency depends on the following factors: (a) interstitial fluid pressure (IFP) within the tumor, (b) local hypoxia, (c) structural abnormalities within the tumor, (d) heterogenous distribution due to abnormal vessel formation within the tumor, and (e) extracellular matrix (ECM), which consists of collagen, fibroblasts, tumor cells and elastin.[29] Before designing a drug for intratumoral administration, we should consider first the method of diffusion that we want to use. The passive transportation, which is based on the physicochemical properties of the injected compound, and active transportation, which is based on the concept of antigen–antibody connection.[30] In addition, within the process of drug administration, heating and cooling techniques have been additionally used to enhance the drug diffusion.[31,32] The time release effect has a major role in this kind of treatment as it prolongs the local deposition to the target tissue and increases apoptosis. Additionally, and at the same time,

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it will postpone any unnecessary toxic drug concentration to diffuse within normal tissue. Carriers have been investigated in order to create a local sustain release effect.[33,34] Nanocarriers have displayed the enhanced permeability and retention (EPR) effect where a drug has increased local deposition and diffusion.[29,35] Surface modification on nanoparticles (NPs) with didodecyldimethylammoniumbromide is an example where the NPs presented greater interaction with the membrane lipids of cancer cells and improved local retention of the administered compound.[36] The EPR effect has been observed to be controlled by heat-shock protein 32 and carbon monoxide.[37] Moreover, the addition of polyethylene glycol (PEG) has been observed to enhance the EPR effect and sustain release as it cannot be recognized by the macrophages.[38] Novel techniques of intratumoral inflammation imaging have been investigated with ^{19}F-magnetic resonance.[39] Currently, there has been extensive research on intratumoral gene therapy in pancreatic cancer, and most of our knowledge regarding this treatment is due to this type of cancer treatment experimentation.[40,41] [11]Intratumoral chemotherapy has been also used for prostate cancer, glioblastoma, melanoma, breast cancer, neuroblastoma and hepatocellular carcinoma[42–50] (Table 1). Several vectors have been used in these different studies, with different intratumoral therapeutic strategies (Table 2). In the current study, we will present our data from the administration of the 2-Diethylaminoethyl-Dextran Methyl Methacrylate Copolymer Non-Viral Vector (DDMC, Ryujyu science corporation, Seto-City, Japan) conjugated with plasmid p53 in C57BL/6 mice in three different groups: (a) control, (b) intravenous, and (c) intratumoral in an effort to identify which methodology could efficiently present local tumor control and distant metastasis control.

RESULTS

Tumor growth rate was controlled in the intravenous and intratumoral group, in comparison to the control group. (Tables 3–5) Our results indicate that distant metastasis in the lung was controlled in a higher degree in the intratumoral group (group 2); in two subjects there were no lung metastasis after 21 days and 6 administrations. In Figure 1, macroscopical findings indicate that only in the control group lung metastasis were visible. In Figure 2, the gene complex is clearly demonstrated within lung micrometastasis for both the intravenous and intratumoral group, therefore it is clear that with both modalities the therapy is efficiently delivered in the lung. However; a higher degree of apoptosis is observed with the intratumoral group as there are clear regions surrounding the gene complex. Also, it has to be stated that in the intravenous group two mice died after administration probably due to the gene complex. The mean survival can be displayed as follows in terms of efficiency: intratumoral (17.4 days) > intravenous (12.6 days) > control (12.6 days). Additionally, Ki-67 and TTF-1 were positively expressed (Figure 3). There was no difference in the survival between the intravenous and control group; however, distant lung metastasis was controlled up to a degree.

DISCUSSION

Previously, it has been observed that local administration of intratumoral chemotherapy is safe and efficient. It was observed that adverse effects were minimal and even complete lung atelectasis was re-expanded.[16] However; the ideal methodology still has to be investigated as several parameters have to be improved. An algorithm has to be built identifying the proper molecules that will efficiently diffuse within the tumor. There are several factors influencing the distribution as previously stated (for example, ECM, IFP, vessel structure) that differ among different tumor types (for example, cavitation-squamus versus no cavitation-non-squamus).[51] The proper volume/concentration that induces cell apoptosis has to be identified for each drug before study initiation. One of the methods that could be used towards this direction is the ITASSER (http://zhanglab.ccmb.med.umich.edu, Ann Arbor, MI, USA), which has already been used in previous studies.[52,53] The same principals of local intratumoral therapy design apply for gene therapy. We would like to have a vector–gene complex that will efficiently distribute

Table 1. Intratumoral gene therapy vectors and cancer types

Author	*Vector*	*Cancer type*	*Ref.*
Hecht *et al.*	TNFerade (AdGVEGR, TNF.11D)	Pancreas	40
Hanna *et al.*	BC-819	Pancreas	41
Li *et al.*	oHSV-1-NIS	Prostate	45
Leifler *et al.*	Adenovirus carrying TIMP-1 or MMP-9	Breast	42
Peng *et al.*	miRNA or shRNA-against target gene (Beclin 1)	Hepatocellular	43
Weibel *et al.*	GLV-1h68	Different tumor models	39
Puntel *et al.*	HC-Ad-TK/TetOn-Flt3L	Glioblastoma	47
Hallet *et al.*	Anti-MMP-9 DNAzyme	Breast	46
Chen *et al.*	PDMSCs-PEDF	Melanoma	44
Xie *et al.*	Ad-IFN-γ	Pancreas	11
Yang *et al.*	Hu 14.18-IL-2	NXS2 neuroblastoma cell line	31
Kasai *et al.*	MGH2.1-CPA-CYP2B1 and CPT11-shiCE	Glioma cells	50
Ramachandran *et al.*	HP-NAP, Ad5PTDf35-[Δ24-sNAP]	Neuroendocrine	49
Huang *et al.*	shVEGF-DOX-dtACPP	Glioma	48

Abbreviations: Ad-IFN-γ, adenovirus-interferon-γ; BC-819, a plasmid comprised of the H19 gene regulatory sequences; DOX, doxorubicin; dtACPP, nanoparticle; GLV-1h68, Vaccinia virus strain; HC-Ad-TK/TetOn-Flt3L, adenoviral vectors encoding cytotoxic herpes simplex type 1 thymidine kinase and the immunostimulatory cytokine fms-like tyrosine kinase ligand 3; HP-NAP-Ad5PTDf35-[Δ24-sNAP], *Helicobacter pylori* neutrophil-activating protein, which mediate antitumor effects by recruiting neutrophils and inducing Th1-type differentiation in the tumor microenvironment; Hu 14.18-IL-2, an immunocytokine consisting of human interleukin-2 linked to hu14.18 mAb, which recognizes the disialoganglioside; MGH2.1, a herpes simplex oncolytic virus type 1 expressing two prodrug-activating transgenes: (a) cyclophosphamide activating P4502B1 and (b) CPT11-activating secreted human intestinal carboxylesterase; MM9, matrix metalloproteinase-9; oHSV-1-NIS, oncolytic herpes simplex virus type 1 with gene coding for human sodium iodide symporter (NIS); PDMSCs-PEDF, placenta-derived mesenchymal stem cells loaded with ecombinant adenoviruses expressing pigment epithelium-derived factor; shVEGF, interfering RNA targeting vascular endothelial growth factor; TIMP-1, tissue inhibitor of metalloproteinase-9; TNFerade (AdGVEGR, TNF.11D), a replication-deficient adenoviral vector that expresses tumor necrosis factor-α.

Table 2. Intratumoral studies with different approaches

Author	*Methodology*	*Subjects*	*Cancer cells–tissue*	*Response*	*Nanoparticles*	*Carriers*	*Ref.*
Horev-Drori *et al.*	^{224}Ra-loaded wires plus gemcitabine/5-FU	*In vitro/in vivo*	Pancreas	√	—	—	77
Xie *et al.*	^{64}Cu-nanoshells	Nude rats	Head–neck	√	√	Nanoshells	11
Hecht *et al.*	TNFerade (AdGVEGR.TNF.11D)	Patients	Pancreas	√	—	—	40
Govindarajan *et al.*	TMAF	*In vitro/in vivo*	Breast–ovarian	√	—	—	52
Lin *et al.*	Review	Review	Review	Review	Lipid nanoparticles	Review	33
Zheng *et al.*	ICG-PL-PEG-mAb	*In vitro/in vivo*	U87-MG human glioblastoma cancer cells	√	ICG-PL-PEG-mAb	PL-PEG	18
Luo *et al.*	Core-loaded fibers with hydroxycamptothecin	*In vitro/in vivo*	H22 hepatoma cells	√	—	Fibers	58
Yang *et al.*	Hu14.18-IL-2	*In vitro/in vivo*	NXS2 neuroblastoma cell line	√	—	—	31
Peiris *et al.*	Three nanoparticle Magnetic chain with doxorubicin	*In vitro/in vivo*	MAT B III tumor-bearing animals	√	Nanochain magnetic particles	—	56
Hanna *et al.*	BC-819	*In vitro/in vivo*	Pancreas	√	—	—	41
Liu *et al.*	mPEG-PCL-Docetaxel	*In vitro/in vivo*	H22 hepatoma cells	√	mPEG-PCL	Poly (caprolactone)	57
Luo *et al.*	PELA Fibers plus hydroxycamptothecin	*In vitro/in vivo*	H22 hepatoma cells	√	PELA	Poly($_{D,L}$-lactide)	58
Geletneky *et al.*	Parvovirus H-1	*in vivo*	Glioblastoma multiforme	√	—	—	17
Zhao *et al.*	NLP-PEG, CLP-PEG plus DOX	*In vitro/in vivo*	H22 hepatoma cells	√	DOX-NLPs, DOX-CLPs, DOX-NLP-PEG, DOX-CLP-PEG	Cationic liposomes, nano lipid particles	38
Ahmed *et al.*	Nanoparticles and Thermal ablation	Review	Review	Review	Review	Review	78
Betting *et al.*	CpG plus rituximab/ cyclophosphamide	*In vitro/in vivo*	B-cell lymphoma	√	—	—	81
Son *et al.*	Dendritic cells plus Cyclophosphamide/ irradiation	*In vitro/in vivo*	CT-26 colon carcinoma cell line	√	—	—	79
Raut *et al.*	Sorafenib	Patients	Refractory sarcomas	√	—	—	80
Li *et al.*	oHSV-1-NIS	*In vitro/in vivo*	Prostate	√	—	—	41
Leifler *et al.*	Adenoviruse carrying TIMP-1 or MMP-9	*In vitro/in vivo*	Breast	√	—	—	42
Peng *et al.*	miRNA or shRNA-against target gene (Beclin 1)	*In vitro/in vivo*	Hepatocellular	√	—	—	43
Weibel *et al.*	GLV-1h68	*In vitro/in vivo*	Different tumor models	√	—	—	39
Puntel *et al.*	HC-Ad-TK/TetOn-Flt3L	*In vitro/in vivo*	Glioblastoma	√	—	—	43
Hallet *et al.*	Anti-MMP-9 DNAzyme	*In vitro/in vivo*	Breast	√	—	—	46
Chen *et al.*	PDMSCs-PEDF	*In vitro/in vivo*	Melanoma	√	—	—	44
Xie *et al.*	Ad-IFN-γ	*In vitro/in vivo*	Pancreas	√	—	—	34
Kasai *et al.*	MGH2.1-CPA-CYP2B1 and CPT11-shiCE	*In vitro/in vivo*	Glioma	√	—	—	50
Ramachandran *et al.*	HP-NAP, Ad5PTDf35-[Δ24-sNAP]	*In vitro/in vivo*	Neuroendocrine	√	—	—	49
Huang *et al.*	shVEGF-DOX-dtACPP	*In vitro/in vivo*	Glioma	√	dtACPP	—	48

Abbreviations: Ad-IFN-γ, adenovirus-interferon-γ; AdGVEGR.TNF.11D, a replication-deficient adenoviral vector that expresses tumor necrosis factor-α (TNF-α); B16, melanoma cell line; B16F10, murine metastatic melanoma in the tails of C57BL/6 mice; BC-819, a plasmid comprised of the H19 gene regulatory sequences; CLP, cationic liposomes; CT-26, colon carcinoma cell line; DOX, doxorubicin; dtACPP, nanoparticle; FU, fluorouracil; GLV-1h68, Vaccinia virus strain; H22, hepatoma cells; HC-Ad-TK/TetOn-Flt3L, adenoviral vectors encoding cytotoxic herpes simplex type 1 thymidine kinase and the immunostimulatory cytokine fms-like tyrosine kinase ligand 3; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HP-NAP-Ad5PTDf35-[Δ24-sNAP], *Helicobacter pylori* neutrophil-activating protein, which mediate antitumor effects by recruiting neutrophils and inducing Th1-type differentiation in the tumor microenvironment; HT29, human colon carcinoma cell lines; Hu14.18-IL-2, an immunocytokine consisting of human IL-2 linked to hu14.18 mAb, which recognizes the GD2 disialoganglioside; ICG-PL-PEG-mAb, indocyanine green-polylactic-polyethylene glycol-integrin α(v)β(3) monoclonal antibody; MAT B, animals inoculated with Mat B-III-uPAR cells; MGH2.1, a herpes simplex oncolytic virus type 1 expressing two prodrug-activating transgenes: (a) cyclophosphamide-activating P4502B1 and (b) CPT11-activating secreted human intestinal carboxylesterase; MM9, matrix metalloproteinase-9; mPEG-PCL, poly(caprolactone); NLP, neutral liposomes; NXS2, neuroblastoma cell line; ODN, oligodeoxynucleotide; oHSV-1-NIS, oncolytic herpes simplex virus type 1 with gene coding for human sodium iodide symporter (NIS); PDMSCs-PEDF, placenta-derived mesenchymal stem cells loaded with ecombinant adenoviruses expressing pigment epithelium-derived factor; PEG, polyethylene glycol; PELA, poly(D,L-lactide); PL, polylactic; shVEGF, interfering RNA targeting vascular endothelial growth factor; SLC-Fc, secondary lymphoid tissue chemokine-Fc; TNFerade, a replication-deficient adenoviral vector that expresses tumor necrosis factor-α; TIMP-1, tissue inhibitor of metalloproteinase-9; U-87-MG, human glioblastoma-astrocytoma, epithelial-like cell line.; - Reproduced with permission from Hohenforst-Schmidt *et al.*[16]

Table 3. Subjects administration log

Day	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	1.10	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	2.10
1																														
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14			1		1 +2			3		1 +2																				
15														1 +2				2		1 +2					0				0	
16							5 +2				a				a		2 +3		a											
17	7 +4																					2	3 +2						2	
18																														
19																														
20																										0				
21																						2		0				2 +3		

Numbers in red indicate the administration date. The ● represents the day of death and number of lung metastasis in one or both. [a]Multiple metastasis in both lungs. Mean survival. Control: 12.6 days. Group 1 (intravenous): 12.6 days. Group 2 (intratumoral): 17.4 days.

Table 4. Surgically resected tumor tissue after death

	Tumor volume in mm^3	*Tumor weight in grams*
Control		
1	44 × 25	8.3
2	23 × 15	3.2
3	34 × 26	6.3
4	30 × 21	3.3
5	43 × 32	10.5
6	40 × 28	9.8
7	39 × 27	9.5
8	34 × 26	6.5
9	27 × 18	6.7
10	42 × 24	9.8
Group 1 (intravenous administration)		
1	29 × 26	8.3
2	12 × 8	1.1
3	13 × 12	2.1
4	27 × 22	6.6
5	28 × 18	5.5
6	12 × 11	1.5
7	26 × 19	7.8
8	26 × 18	6.9
9	26 × 18	7.2
10	28 × 22	8.1
Group 2 (intratumoral administration)		
1	22 × 20	9
2	30 × 23	9.6
3	28 × 24	8.6
4	31 × 22	6.8
5	28 × 25	5
6	28 × 21	6.7
7	22 × 18	4.5
8	31 × 24	9.2
9	30 × 25	8.3
10	29 × 26	8.7

Table 5. Tumor measurement from experiment initiation and every 7 days

	First measurement	*Second measurement*	*Third measurement*
C1	7.5 × 5.8 (126.15)	25.6 × 12.1 (1874.05)	42.4 × 23.5 (11 707.7)
C2	10 × 9.3 (432.45)	23.2 × 15.3 (2715.44)	
C3	9.2 × 4.7 (101.61)	24.1 × 19.3 (4488.5)	34.3 × 26.5 (12 043.59)
C4	6.3 × 3.2 (32.26)	21 × 17.5 (3215.63)	
C5	10.3 × 7.4 (282)	29.4 × 22.3 (7310.16)	43.2 × 32.7 (23 096.66)
C6	6.5 × 3.4 (37.57)	21.7 × 18.1 (3554.57)	38.1 × 27.1 (13 990.51)
C7	6.4 × 3.8 (46.2)	21.9 × 17.6 (3391.87)	37.7 × 26.2 (12 939.39)
C8	9 × 4.2 (79.38)	23.8 × 18.9 (4250.8)	33.9 × 25.7 (11 195.3)
C9	6.1 × 3 (27.45)	20.1 × 17.2 (2973.19)	
C10	9.7 × 4.4 (93.9)	24.4 × 19 (4404.2)	41.7 × 23.6 (11 612.62)
1.1	6.2 × 3.7 (42.44)	19.1 × 18.5 (3268.49)	27.2 × 25.8 (9052.7)
1.2	11.3 × 5.4 (164.75)		
1.3	12.3 × 7.2 (318.82)	12.8 × 12.2 (952.58)	
1.4	9.1 × 6.3 (180.59)	13.8 × 13 (1166.1)	26.6 × 22 (6437.2)
1.5	5.7 × 4.8 (65.66)	13 × 7.5 (365.63)	25.8 × 17.5 (3950.63)
1.6	11.2 × 6.4 (229.38)	11.5 × 10.8 (670.68)	
1.7	5.5 × 4.4 (53.24)	12.2 × 8.1 (400.22)	24.9 × 18.2 (4123.94)
1.8	10.5 × 5.2 (141.96)	13.8 × 8.2 (463.96)	25.9 × 17.8 (4103.08)
1.9	6.2 × 5.1 (80.63)	13.1 × 7.9 (408.79)	25.6 × 17.3 (3830.91)
1.10	9.2 × 6.1 (171.17)	14 × 13.2 (1219.68)	27 × 21.7 (6357.02)
2.1	6.8 × 3.5 (41.65)	20.6 × 15.8 (2571.29)	
2.2	10 × 6.7 (224.45)	19.3 × 15.6 (2348.42)	27.5 × 21.8 (6534.55)
2.3	9.4 × 4.8 (108.29)	16 × 14 (1568)	26.6 × 23.7 (7470.48)
2.4	8.9 × 5.1 (115.74)	16.7 × 15.7 (2058.19)	26.7 × 19.6 (5128.54)
2.5	7.1 × 4.9 (85.24)	20.5 × 14.7 (2214.92)	27.5 × 24.6 (8320.95)
2.6	8.7 × 4.7 (96.09)	16.5 × 15.1 (1881.08)	26.4 × 19.1 (4815.49)
2.7	6.5 × 3.6 (42.12)	20.1 × 15.9 (2540.74)	
2.8	9.8 × 6.3 (194.48)	19.1 × 15.2 (2206.43)	27.3 × 21.5 (6309.71)
2.9	9.1 × 4.9 (109.25)	16.1 × 14.3 (1646.14)	26.8 × 23.9 (7654.21)
2.10	7.3 × 5.2 (98.7)	20.7 × 14.9 (2297.8)	27.8 × 24.8 (8549.06)

Tumor volume measurements in mm^3. Number in parenthesis represents volume measurement after the additional equation ½(length × width^2) σε mm^3. In groups 1 and 2, tumor growth rate is reduced in comparison to the control.

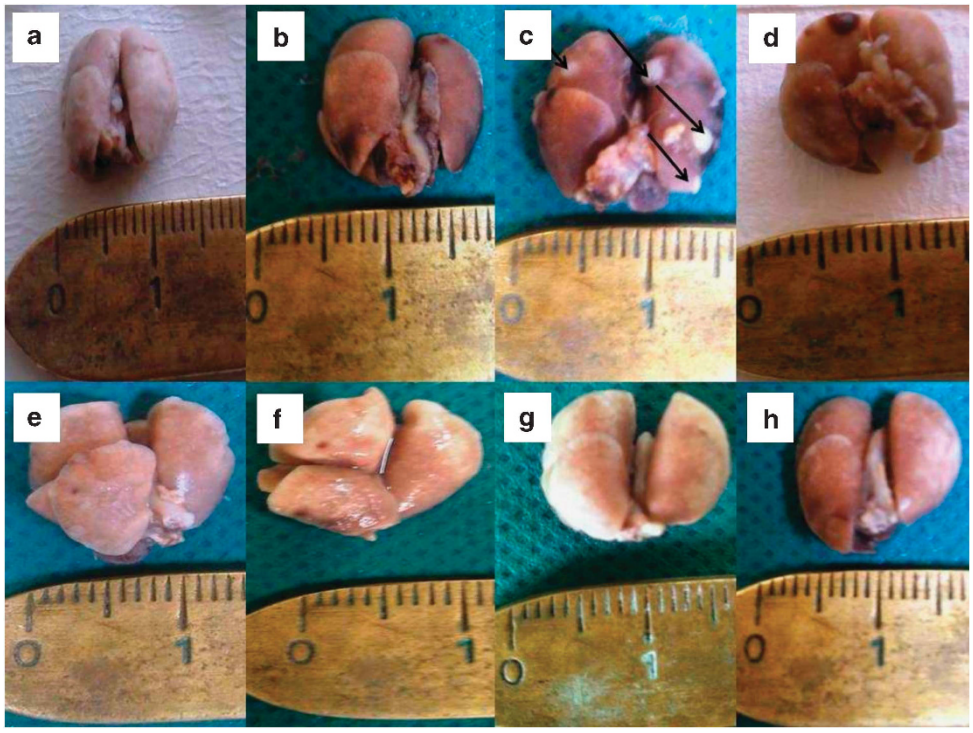

Figure 1. Macroscopic appearance of lung in different groups (**a**–**c**: control group, **d**–**f**: intravenous group, **g** and **h**: intratumoral group). Black arrows indicate macroscopic lung metastasis. Macroscopic surface metastases were observed only in lungs of the control group (panel **c**: black arrows).

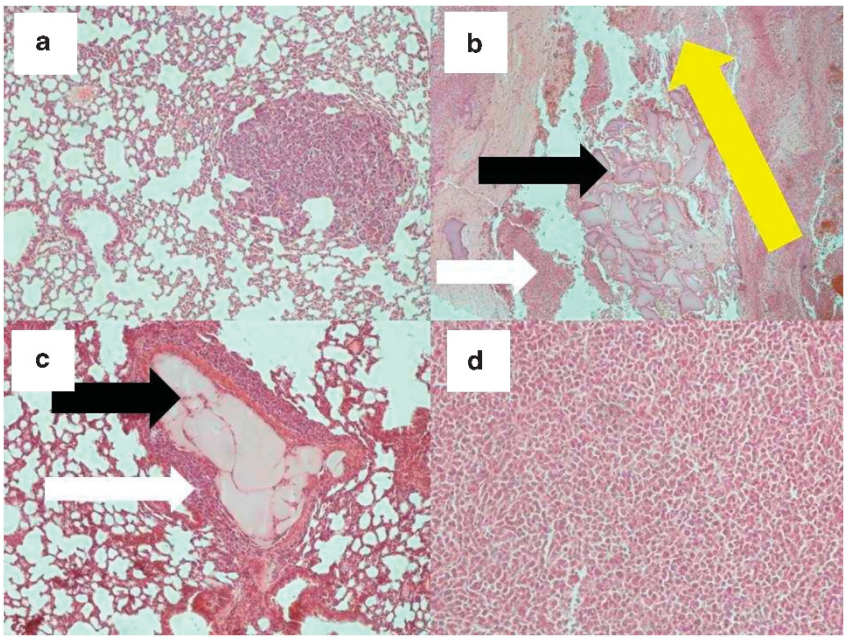

Figure 2. (**a**) Lung micrometastasis control group; (**b**) yellow arrow indicates root of intratumoral injection and gene-complex release, white arrow indicates tumor necrosis and black arrow indicates the gene-complex (**c**) black arrow indicates the gene complex and white arrow tumor necrosis; (**d**) primary tumor cells (back inoculated tumor).

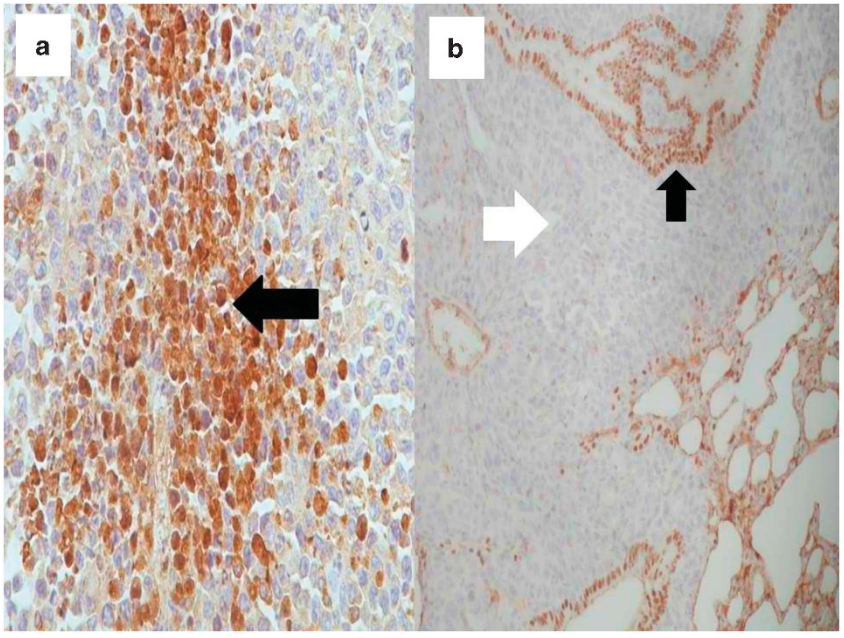

Figure 3. (**a**) Black arrow represents Ki67 expression; (**b**) white arrow represents the tumor and black arrow represents the TTF-1.

within the tissue and if possible through local vessels and lymphnodes throughout the systematic circulation.[54] This observation has been done with aerosol local chemotherapy administration where distribution of the administered drug was observed in the local lymph nodes and local cisplatin concentration was correlated with systematic.[54,55] It has been previously stated that rapid tumor cell proliferation and weakly developed lymphatics cause high IFP and blood vessel remodeling by intus-susception or compression.[51] Therefore high interstitial pressure is observed in the center of the tumor, which blocks the efficient distribution of the drug, whereas this effect is diminished while moving from the center of the tumor to the periphery. The ECM differs between normal tissue and cancer tissue. The following collagen types I, II, III, V and IX, tenasin C, fibronectin and proteoglycans exhibit increased accumulation and generate a dense network in tumor tissues. Moreover, excessive deposition of ECM components decrease the distance between neighboring ECM components and diminish the pore size of the tumor matrix. Increased 'stiffness' of the ECM in cancer tissue is observed and therefore the efficient distribution is again blocked for various molecules such as; anti-tumor immune cells, chemotherapeutic agents, therapeutic viruses, immunotoxins, interferons, monoclonal

Figure 4. Insoulin syringe 1 ml (100 Units) and 27-gauge needle.

antibodies and complement.[51] First, ECM influences the IFP. Furthermore, the abnormal architecture of vessels and lymphatics are responsible for blocking the defense mechanisms of the body, such the M2 macrophages. The intravenous-administered drugs once administered reach the tumor sites and exit the tumor vasculature and translocate through the interstitial space in order to reach their target cells. Trans-endothelial transport of macromolecular drugs involves a phenomenon known as the EPR effect in solid tumors.[51] We need the EPR effect for the leaky abnormal vessels within the tumor to enhance the different macromolecule distribution. The EPR effect is enhanced with novel nanocarriers.[33,38,48,56–58] It has been previously observed that the hyper-permeability of the tumor vessels in combination with the absence of functional lymphatics induce a prolonged deposition of several drugs. The hyper-permeability (>10 nm) allows drug molecule's transportation within the tumor tissue; however, not in the normal tissue where particles >10 nm cannot be transported. Therefore this effect can be used as a method of normal tissue protection. It has to be stated that the EPR effect differs between cancer types and within the tumor from one region to another.[59] The tumor tissue matrix is a very important parameter; dense extracellular fibers and matrix within the tumor will block large NPs to efficiently penetrate the tumor and diffuse.[60,61] Renal clearance is more rapid in smaller NPs (<6 nm), while reticuloendothelial clearance is usually avoided with PEGylated drug, like in the case of pegylated liposomal doxorubicin.[62,63] Moreover, the shape and charge of NPs have an important role in the diffusion efficiency. Elongated NPs penetrate the vascular flux more efficiently when compared with spherical particles.[64] Cationic NPs transported more efficiently when compared with neutral or anionic.[65,66] Novel nanoparticles are designed to decrease their size upon acidic pH and matrix metalloproteinases (MMPs), however; further experimentation is needed in order to draw a clear conclusion how these parameters interact with the tumor microenvironment.[67,68] The IFP is high within solid tumors and inhibits the penetration of drugs.[69] Increased IFP is also due to a dense ECM and inadequate lymphatic drainage.[70] Again increased IFP inhibits drug penetration within solid tumors. High levels of hyaluronic acid (HA) have been found in the ECM of solid tumors and are collated with increased IFP. Administration of HA-targeting enzyme (PEGPH20) was able to diminish the HA levels and therefore vessels were patent and drug penetration was efficient.[71] Furthermore, upon designing the study we should know how the administered solution will be diffused throughout the target tissue. Positron emission tomography is one method previously used to identify the optimal volume/concentration for intratumoral administration.[72] There are two major methods of transportation: the passive and active targeting. The active transportation is based on the ligand–receptor interaction, while in the passive transportation the diffusion of a compound within the tissue is based on its physical properties.[30] Gene therapy has been previously investigated targeting epidermal growth factor, vascular endothelial growth factor, KRAS, immunotherapy, ECM factors and tumor microenvironment.[42,48,49,73–76] Additional methods of enhancing the intratumoral gene therapy have been previously performed with the addition of radiotherapy, chemotherapy, thermal ablation, sorafenib, imatinib, use of ultrasound system, rituximab and dendritic cells to gene therapy administration alone.[34,77–82] In our current study, we used the novel non-viral vector DDMC as the vehicle for the local intratumoral administration of pSicop53. The DDMC was synthesized by graft polymerization of methyl methacrylate (MMA) onto 2-Diethylaminoethyl-Dextran Methyl Methacrylate Copolymer (DAEX). These copolymers have hydrophobic and hydrophilic regions and have high transfection efficiency and they can also be sterilized by autoclavation.[83] Investigation with DDMC/DNA presented *in vitro* higher transfection efficiency in COS-7 cell lines[84] when it compared with DAEX/DNA in HEK293 cell lines.[85] DDMC has efficient absorption capability both for RNA and DNA. This is due to their cationic property and has been found to be influenced by pH and ionic strengths.[86] Furthermore, the DDMC/DNA formation reaction is influenced by the Coulomb forces. The hydrophobic bonding strength as well as the hydrogen bonding strength have a role due to the hydrophobicity of the grafted MMA sections. Optimal cell affinity was also previously observed.[87] The DDMC/DNA and gene transfection are still under investigation.[83]

CONCLUSIONS

Intratumoral gene therapy can be used alone or in combination with additional methods, such as radiotherapy and/or chemotherapy. Gene therapy could be used to sensitize chemo-resistant or radio-resistant tumors during the treatment course. The application currently can be done in lesions visible within the respiratory tract or using the endobronchial ultrasound bronchoscope. It is an efficient method of treatment; however, current studies indicate that a combination with additional modalities as previously stated offer improved disease control. Intratumoral gene therapy for lung cancer still has to find its place in the algorithm of treatment either as neo-adjuvant in early-stage disease or as a palliative in advanced stages.

MATERIALS AND METHODS

Non-viral vector and p53

The non-viral vector was purchased from Ryujyu science corporation, Seto-City, Japan by PZ and AB under the contract EG179806487JP (A18503015(121223b1), A18503016(121227b3), A18503017(121227b4), A18503018(121227b5), A18503019(130228b8) and A18503020(130228b10)). The non-viral vector has the following characteristics: fast and easy procedure, stable for autoclaving sterilization at 121 °C for 15 min, broad peak performance, applicable in high-throughput screening, no serum inhibition, broad cell line range, best results with siRNA applications, excellent reproducibility, low toxicity in comparison with DEAE-dextran, high efficiency by use of low DNA amounts, a high DNase protection facility by DNase degradation, and best price/value ratio. The plasmid p53 was purchased from Addgene (Cambridge, MA, USA) as 'Addgene plasmid 123519, 124665, 125156,125157'. Enhanced green fluorescent protein is expressed from this plasmid as a marker, but it is not a fusion protein. Cre causes enhanced green fluorescent protein to be recombined out of the construct, activating shRNA expression (Vector backbone: pSico, Vector type: Mammalian Expression, Lentiviral, RNAi, Cre/Lox).[88] The preparation of the complex (non-viral vector-p53) has been previously described, and 0.2 ml was chosen to be the injected volume for both the investigated groups.[89,90]

Mice

Thirty C57BL/6 mice aged 7–8 weeks were purchased from the Hellenic Institute (Athens, Greece) PASTEUR (code 000.2481) with purchase code A-ΔA00000399 and were divided into three groups. The Institute has the following authorization for production and experimentation of mice EL 25 BIO 011 and EL 25 BIO 013. The mice included were isolated (one per cage) in a temperature-controlled room on 12-h light–dark cycle and were allowed free access to food and water. The Lewis lung carcinoma cell line was obtained from ATCC (LGC Standards GmbH, Wesel, Germany) (CRL-1642). The cells were routinely cultured in 25-cm^2 tissue culture flasks containing RPMI (ATCC, 30-2002) supplemented with 10% fetal bovine serum (Biochrom, Thessaloniki, Greece) according to the supplier's instruction. The cell line was incubated at 37 °C in 5% CO_2. The doubling time of the cell line was 21 h.[91] At confluence, cells were harvested with 0.25% trypsin and then were resuspended at 1.5×10^6 cells in 0.15 ml phosphate-buffered saline, Dulbecco, Biochrom), which was injected in mice. The back was inoculated subcutaneously (27-guage needle). The tumor volume was measured once weekly using bidimensional diameters (caliper) with the equation $V = 1/2ab^2$, where the a represents the length and b the width (mm^3). The tumor was grown on the back of the mice (Figure 4).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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