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Significance of RNA Reference in Tumour-related Gene Expression Analyses by cDNA Array

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Abstract. *The cDNA array technique is an efficient approach for studying the expression of a large number of genes in a single experiment. The cDNA array analysis indicates the relative level of corresponding gene expression from a specimen and a reference. Our investigation was performed to address the significance of reference RNA on the outcome of the cancer-related gene expression profile obtained from cDNA array analysis. Human head and neck squamous cell carcinoma (HNSCC) biopsies and 5 sources of RNA reference were used for this purpose. In these biopsies, each individual patient expressed a unique set of genes both in normal and tumour tissue. It is important to note that 5 striking patterns of tumour-related gene expression were obtained according to the 5 references used. Significant differences in 60%, 16%, 15% and 15% of the genes expressed were shown when autologous normal matched tissue biopsy references were compared to pooled cell lines, allogenic normal mixed cell types, tumours or allogenic normal matched cell type references, respectively. Thus, theoretically and our study suggested that patient autologous normal cells matching with the tumour type should be the most suitable reference in cDNA array for the identification of individual tumour gene profiles with clinical purpose.*

It is well established that genetic alteration is involved in all stages of tumour development. The identification of tumour-related genes could potentially lead to improved diagnosis and facilitate the choice of the most effective therapeutic strategies. The alteration of tumour-related gene expression compared to the autologous normal cell counterpart needs to be established.

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Unlike conventional methods for gene expression profiling by which only one or a few genes can be analysed, the cDNA array technique is an efficient approach for studying the expression of a large number of genes in a single experiment. This approach has been used to assess the molecular contributors to biological processes and in the classification of human cancers, where histopathology or standard clinical indicators were inadequate (1). The cDNA array analysis indicates the relative level of corresponding gene expression from a specimen and a reference. Interestingly, various investigators used RNA obtained from pooled allogenic normal cells, *in vitro* permanent growing human-derived cell lines or pooled tumours as reference in cDNA array for analysing tumour- related gene expression (2-6). The tumour-related gene profiles obtained from such experimental settings have been suggested to be the molecular portraits directly linked to physiological variation of the tumour and could be used for diagnosis, prognosis and treatment (3, 7-9).

In order to investigate the significance of the RNA reference on tumour-related gene expression profile, human head and neck squamous cell carcinoma (HNSCC) biopsies were used as the tumour model. Five different RNA sources were used as references. These were autologous normal squamous epithelial biopsies, pooled allogenic normal mixed cell types, pooled human-derived cell lines, pooled HNSCC biopsies or pooled normal squamous epithelial biopsies.

Materials and Methods

HNSCC and autologous normal squamous epithelial biopsies. HNSCC tissues and their autologous unaffected normal squamous epithelial oral mucosal were obtained from 16 patients (Table I). In order to avoid the existence of a malignant subpopulation within the normal phenotype biopsies (10), the malignant and normal cell status were confirmed by DNA content and standard pathological investigation (Data not shown).

The biopsies were immediately snap-frozen at -70˚C upon surgical removal. All biopsies were collected after appropriate

Table I. *Head and neck squamous cell carcinoma (HNSCC) biopsies.*

Patient	Sex	Age	Site	Tumor/Node/ Metastasis	Differentiation
T0213	M	58	Tonsil	T4N2bM0	Poor
T0214	M	71	Tongue	T2N0M0	Moderate
T0215	M	42	Tongue	T3N1M0	Moderate
T0216	M	60	Tonsil	T4N0M0	Poor
N0203	F	78	Tongue	T4N2bM0	Moderate
T0219	F	76	Gingiva	T4N2bM0	Moderate
T0221	M	47	Tonsil	T3N2bM0	Poor
T0224	F	62	Tonsil	T3N0M0	Moderate
T0225	M	79	Floor of mouth	T4N2bM0	Moderate
T0240	F	69	Tongue	T3N01M0	moderate
T0241	M	55	Tonsil	T4N2aM0	Moderate
T0309	M	58	Tongue	T2N0M0	Poor
T0310	M	55	Soft palate	T2N2cMx	Poor
T0313	F	64	Hypopharynx	T2N3M0	Moderate
T0317	M	61	Larynx	T4N0M0	Moderate
T0318	M	53	Tonsil	T4N1M0	Moderate

patient consent and agreement. The Medical Ethical Committee at Huddinge University Hospital, Karolinska Institute, Sweden, approved this study.

Fresh human allogenic normal mixed cell types and cell lines. Blood lymphocytes, fibroblasts, tonsil cells, epithelial cells and adipocytes were obtained from 5 healthy individuals. Ten permanent *in vitro* growing human-derived cell lines, K562(11), SSC4 (12), PGD1(13), Rhek1 (14), FaDu (15), Detroit (16), RL7 (17), DS14I(18), Hek 293 (19) and SH-SY5Y (20), were used.

Total RNA extraction, cDNA probe synthesis and gene expression analysis. At least 10 mg of frozen biopsies, 10 million normal cells or cell lines were homogenised and separated by standard guanidinium thiocyanate and phenol extraction. The integrity of the total RNA preparations was examined by standard gel electrophoresis. RNA concentration and purity were determined at absorbance ratios 260nm/280nm.

Five μg of total RNA was used as template for biotin-labelled cDNA probe synthesis. The relative expression of 100 well-defined genes were analysed using the GEArray Q series (SuperArray Inc., USA). This array contained 300-600 bp cDNA fragments, printed with 1-mm tetraspot format/gene on nylon membrane. Plasmid DNA, pUC18 and blank spots were also included as negative controls to confirm hybridisation specificity of the test. The UniGene/Gene Bank accession number and array position of the genes can be accessed at www.superarray.com. In order to minimise experimental variation, the entire array filters and reagents were purchased from the same batch.

The cDNA probes were hybridised to cDNA fragments on the GEArray membranes. The chemiluminescence signal intensity of each gene was obtained by exposing the hybridised filter to X-ray film. In order to obtain the comparable value, normalisation of the signal intensity of each gene to the housekeeping gene, ribosomal protein L13A (RPL13A) on the same membrane was done. After being normalised with RPL13A, the given genes were considered as up- or down-regulated when the ratio of the gene in each tumour was above the two-fold arbitrary threshold over or under the corresponding gene in the reference.

Statistical analysis. Comparisons of gene expression profile in cDNA array from each reference source were performed using a two-tailed Student's *t*-test for paired observation. $P<0.05$ was considered statistically significant. All statistical tests were performed with StatView+Graphic (Abacus, Raleigh, USA).

Results

Gene expression in individual normal squamous epithelial biopsies, HNSCC biopsies, pooled allogenic normal cells and cell lines (Figure 1). With the housekeeping gene ribosomal protein L13A (RPL13A) as the normalising internal control, the level genes expressed in tumour biopsies and the normal cells differed. The variable expression levels and the number of affected genes in the tumour or autologous normal tissue counterpart in each individual patient were unique (Figure 1B and C). The pooled cell lines expressed 70 out of the 100 tested genes (70%), higher than pooled allogenic cells (Figure 1A). Interestingly, only VEGF expression was higher in pooled normal allogenic cells than pooled cell lines. This might suggest the angiogenesis dependence of normal cells and independence of *in vitro* established cell lines.

Gene expression profile of normal cells (Figure 2). After normalisation with RPL13A, four patterns of gene expression could be obtained from individual patient normal squamous biopsies when four references were used. These references were pooled allogenic normal mixed cell types (Figure 2 B), pooled cell lines (Figure 2 C), pooled tumours (Figure 2 D) or pooled patient normal squamous biopsies (Figure 2 E). In comparison, the most down-regulated genes of individual patient squamous epithelial biopsies were detected with pooled cell lines reference (Figure 2C).

Individual tumour-related gene expression profiles (Figure 3, Tables II and III). Five striking patterns of the individual tumour-related gene expression profiles were obtained when 5 different sources of RNA reference were used (Figure 3).

Statistical difference ($p<0.05$) at 16%, 60%, 15% and 15% of the tumour-related gene expression with patient autologous normal squamous epithelial reference compared to pooled allogenic normal mixed cell types (Table II A, B), pooled cell lines (Table II A, C) pooled tumours (Table II A, C) or pooled patient normal squamous epithelial references (Table II A, E), respectively. Thirteen % of the tumour-related gene expression was significantly different ($p<0.05$) when pooled patient normal epithelial tissue

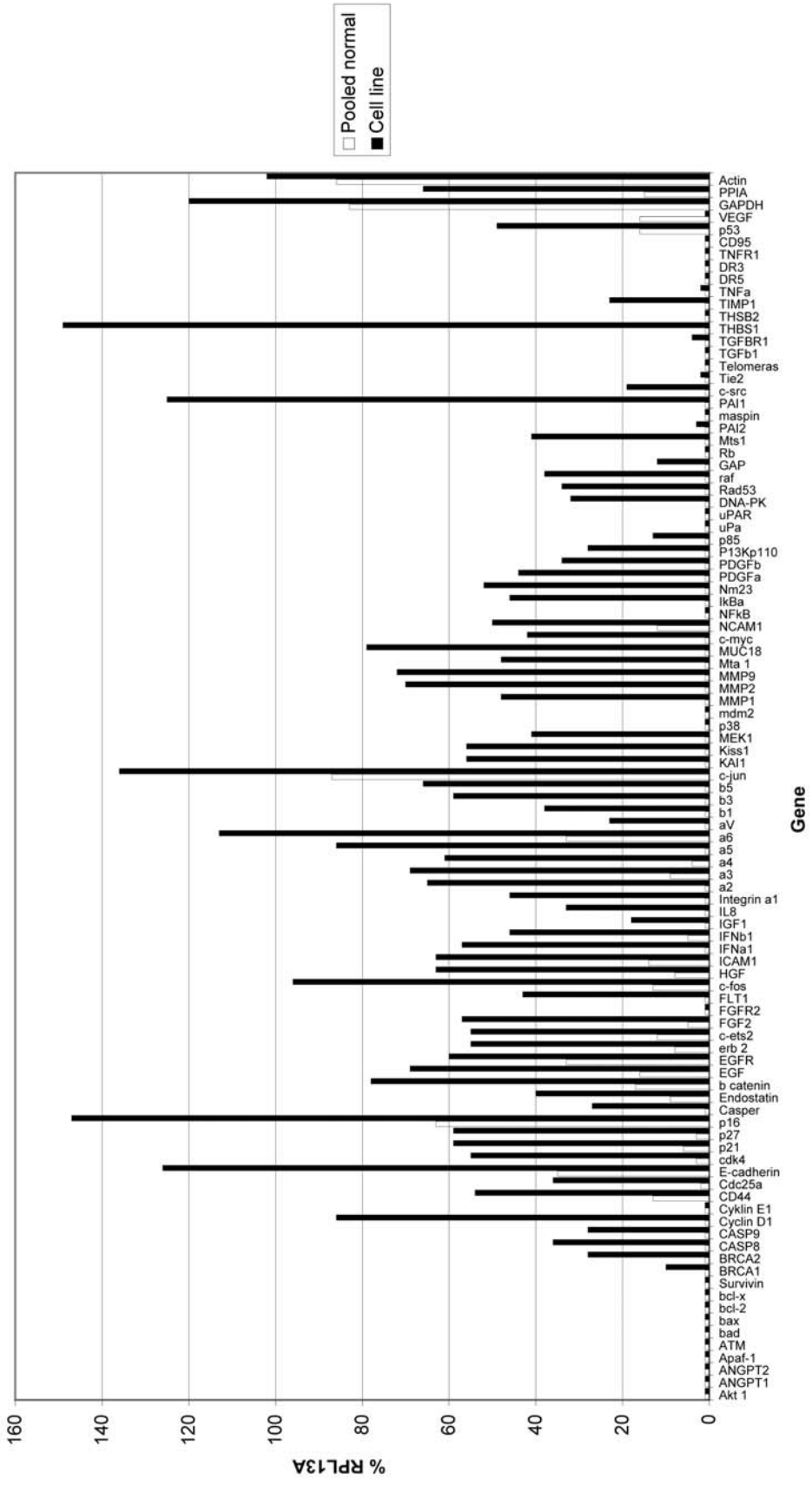
Pooled normal
Cell line
Actin
PPIA
GAPDH
VEGF
p53
CD95
TNFR1
DR3
DR5
TNFa
TIMP1
THSB2
THBS1
TGFBR1
TGFb1
Telomeras
Tie2
c-src
PAI1
maspin
PAI2
Mts1
Rb
GAP
raf
Rad53
DNA-PK
uPAR
uPa
p85
P13Kp110
PDGFb
PDGFa
Nm23
IkBa
NFkB
NCAM1
c-myc
MUC18
Mta 1
MMP9
MMP2
MMP1
mdm2
p38
MEK1
Kiss1
KAI1
c-jun
b5
b3
b1
aV
a6
a5
a4
a3
a2
Integrin a1
IL8
IGF1
IFNb1
IFNa1
ICAM1
HGF
c-fos
FLT1
FGFR2
FGF2
c-ets2
erb 2
EGFR
EGF
b catenin
Endostatin
Casper
p16
p27
p21
cdk4
E-cadherin
Cdc25a
CD44
Cyklin E1
Cyclin D1
CASP9
CASP8
BRCA2
BRCA1
Survivin
bcl-x
bcl-2
bax
bad
ATM
Apaf-1
ANGPT2
ANGPT1
Akt 1
Gene
160
140
120
100
80
60
40
20
0
% RPL13A

Figure 1A.

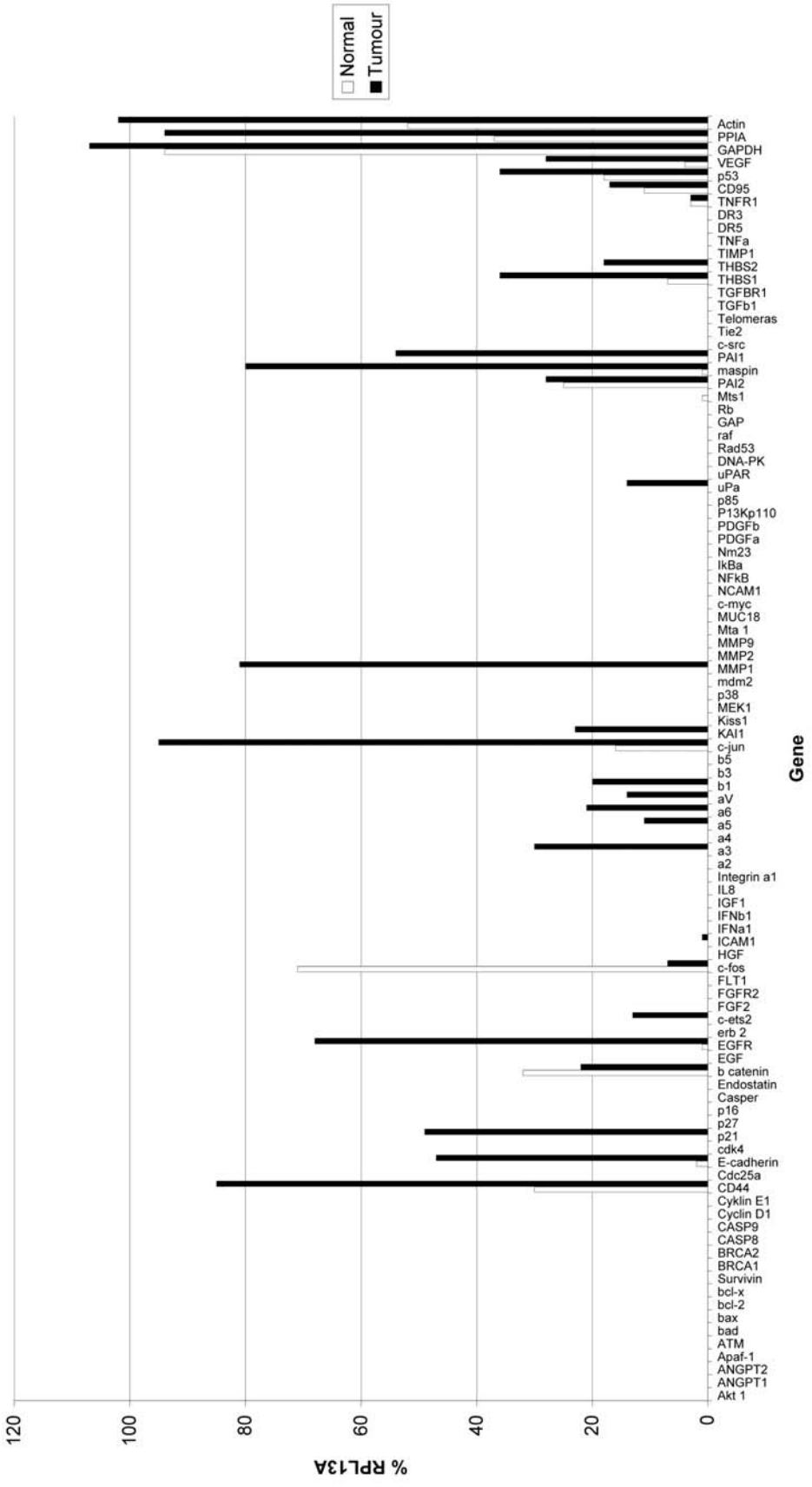
Normal
Tumour
Actin
PPIA
GAPDH
VEGF
p53
CD95
TNFR1
DR3
DR5
TNFa
TIMP1
THBS2
THBS1
TGFBR1
TGFb1
Telomeras
Tie2
c-src
PAI1
maspin
PAI2
Mts1
Rb
GAP
raf
Rad53
DNA-PK
uPAR
uPa
p85
P13Kp110
PDGFb
PDGFa
Nm23
IkBa
NFkB
NCAM1
c-myc
MUC18
Mta 1
MMP9
MMP2
MMP1
mdm2
p38
MEK1
Kiss1
KAI1
c-jun
b5
b3
b1
aV
a6
a5
a4
a3
a2
Integrin a1
IL8
IGF1
IFNb1
IFNa1
ICAM1
HGF
c-fos
FLT1
FGFR2
FGF2
c-ets2
erb 2
EGFR
EGF
b catenin
Endostatin
Casper
p16
p27
p21
cdk4
E-cadherin
Cdc25a
CD44
Cyklin E1
Cyclin D1
CASP9
CASP8
BRCA2
BRCA1
Survivin
bcl-x
bcl-2
bax
bad
ATM
Apaf-1
ANGPT2
ANGPT1
Akt 1
Gene
120
100
80
60
40
20
0
% RPL13A

Figure 1B.

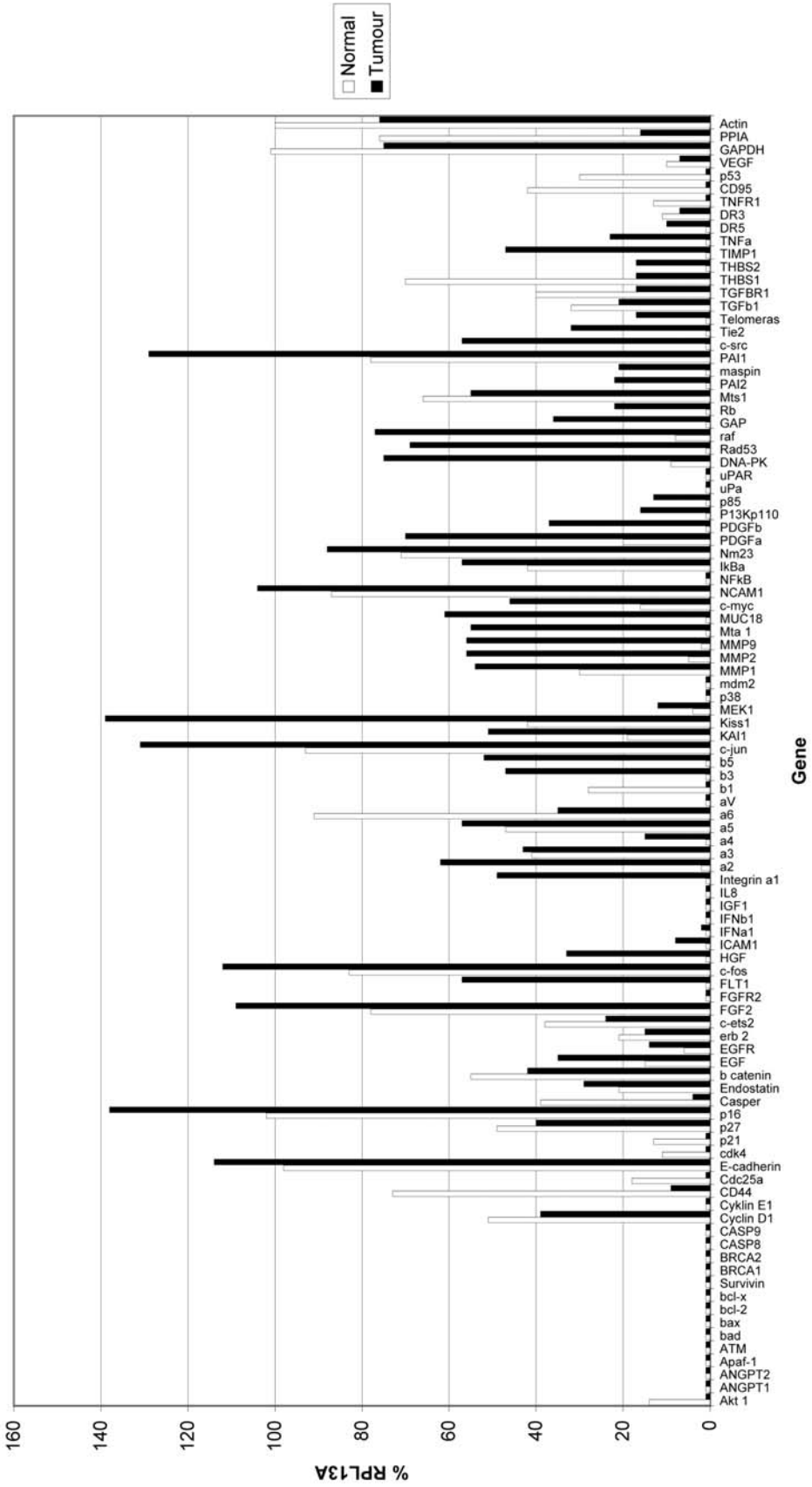

Figure 1C.

Figure 1. *The level of gene expression after being normalised to the housekeeping gene, ribosomal protein L13A (RPL13 A) in the same array filter. (A) Pooled normal cells and pooled cell lines; (B) patient N0203 tumour and autologous normal epithelial biopsy and (C) patient T0215 tumour and autologous normal epithelial biopsy.*

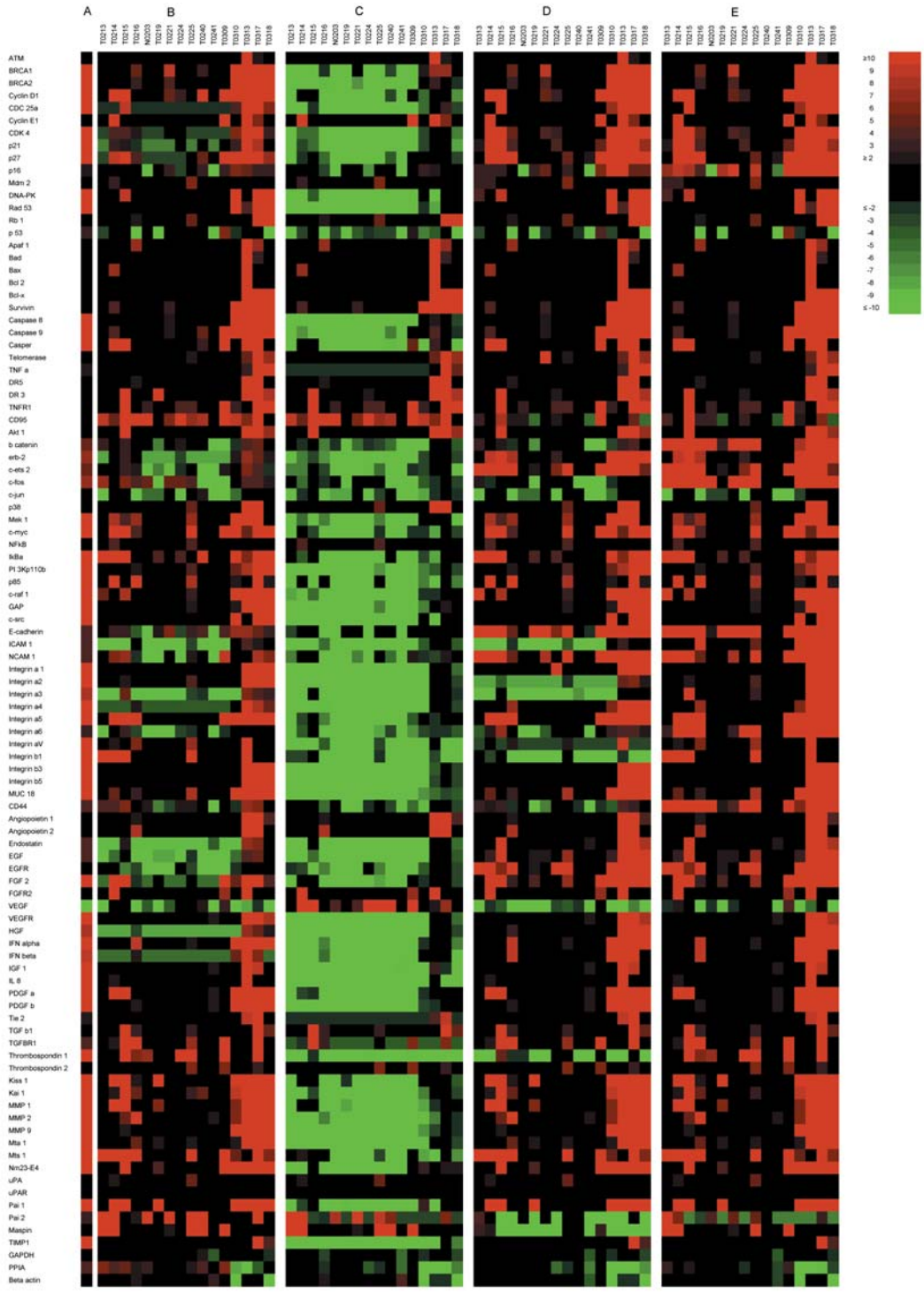

Figure 2. *The ratio of gene expression after being normalised with the housekeeping gene, RPL13A in the pooled cell lines when pooled normal cells were used as reference (A). The ratio of gene expression in normal squamous epithelial biopsies of individual patients when pooled allogenic normal cells (B), pooled cell lines (C), pooled tumours (D) or pooled patient normal squamous epithelial biopsies (E) were used as reference.*
Each column represents an individual patient normal squamous epithelial biopsy and each row represents a single gene. As shown in the colour bar, the intensity of red indicates up-regulation and green indicates down-regulation.

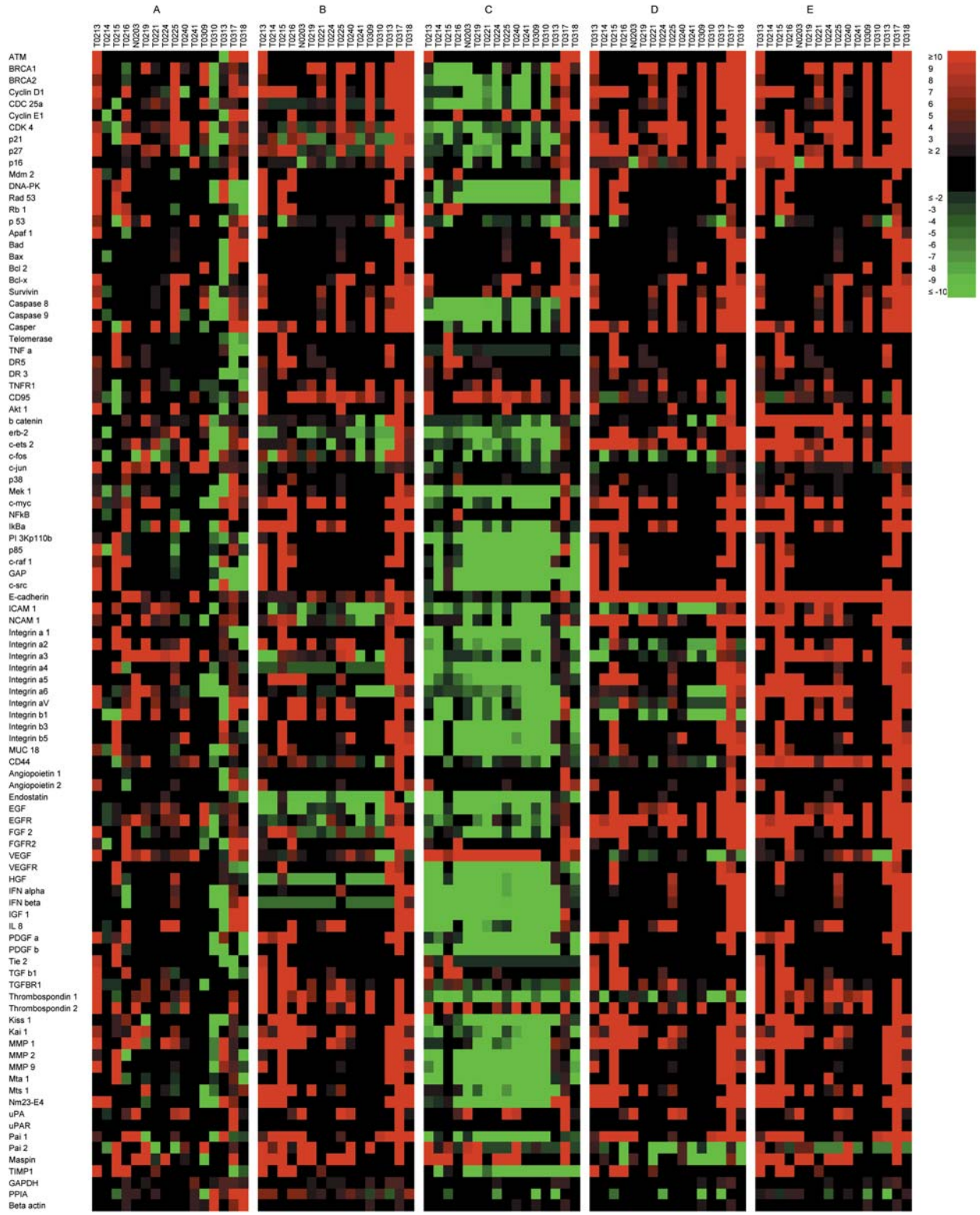

Figure 3. *The ratio of gene expression after being normalised with the housekeeping gene, RPL13A in HNSCC biopsy when the autologous normal squamous epithelial biopsy (A), pooled allogenic normal cells (B), pooled cell lines (C), pooled tumours (D) or pooled patient normal squamous epithelial biopsies (E) were used as reference. Each column represents an individual patient tumour biopsy and each row represents a single gene. As shown in the colour bar, the intensity of red indicates up- regulation and green indicates down-regulation.*

Table II. *Variation of tumor-related gene expression with patient autologous normal epithelial biopsies (A), pooled normal cells (B), pooled cell lines (C), pooled tumours (D) and pooled patient normal biopsies (E) used as reference, were compared. The p -value (p)were obtained by Paired Student's t-test.*

	P(A:B)	P(A:C)	P(A:D)	P(A:E)	P(B:C)	P(B:D)	P(B:E)	P(C:D)	P(C:E)	P(D:E)
ATM	0.4611	0.4611	0.4611	0.4611	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
BRCA1	0.1307	0.4358	0.1307	0.1307	0.1057	1.0000	1.0000	0.1057	0.1057	1.0000
BRCA2	0.1593	0.0006	0.1593	0.1593	0.0474	1.0000	1.0000	0.0474	0.0474	1.0000
Cyclin D1	0.0992	0.0030	0.0992	0.0992	0.0145	1.0000	1.0000	0.0145	0.0145	1.0000
CDC 25a	0.2296	0.0006	0.1702	0.1702	0.0236	0.4781	0.4781	0.0537	0.0537	1.0000
Cyclin E1	0.2700	0.2700	0.2700	0.2700	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
CDK 4	0.2962	0.0027	0.1427	0.1427	0.0086	0.2800	0.2800	0.0388	0.0388	1.0000
p21	0.9088	0.0074	0.1067	0.1067	0.0025	0.1088	0.1088	0.0107	0.0107	1.0000
p27	0.1550	0.0181	0.1244	0.1244	0.0181	0.3025	0.3025	0.0580	0.0580	1.0000
p16	0.4086	0.1904	0.7664	0.0456	0.4026	0.3105	0.0194	0.1566	0.0168	0.0723
Mdm 2	0.9739	0.9739	0.9739	0.9739	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
DNA-PK	0.3098	0.0063	0.3098	0.3098	0.0001	1.0000	1.0000	0.0001	0.0001	1.0000
Rad 53	0.2726	0.0003	0.2726	0.2726	0.0002	1.0000	1.0000	0.0002	0.0002	1.0000
Rb 1	0.3449	0.3449	0.3449	0.3449	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
p 53	0.2186	0.0371	0.1000	0.2043	0.1110	0.3483	0.9211	0.3353	0.1241	0.3973
Apaf 1	0.2961	0.2961	0.2961	0.2961	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Bad	0.3543	0.3543	0.3543	0.3543	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Bax	0.7810	0.7810	0.7810	0.7810	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Bcl 2	0.7928	0.7928	0.7928	0.7928	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Bcl-x	0.1851	0.1851	0.1851	0.1851	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Survivin	0.1615	0.1615	0.1615	0.1615	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Caspase 8	0.2427	0.0002	0.2427	0.2427	0.0443	1.0000	1.0000	0.0443	0.0443	1.0000
Caspase 9	0.2158	0.0008	0.2158	0.2158	0.0809	1.0000	1.0000	0.0809	0.0809	1.0000
Casper	0.2185	0.0031	0.2185	0.2185	0.0699	1.0000	1.0000	0.0699	0.0699	1.0000
Telomerase	0.2670	0.2670	0.2670	0.2670	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
TNF a	0.3458	0.5292	0.3458	0.3458	0.0352	1.0000	1.0000	0.0352	0.0352	1.0000
DR5	0.1828	0.2890	0.1828	0.1828	0.6738	1.0000	1.0000	0.6738	0.6738	1.0000
DR 3	0.0562	0.0562	0.0562	0.0562	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
TNFR1	0.0110	0.0110	0.0110	0.0110	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
CD95	0.0034	0.0034	0.3478	0.2285	1.0000	0.0069	0.0112	0.0069	0.0112	0.6003
Akt 1	0.1894	0.1894	0.1894	0.1894	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
b catenin	0.5644	0.0557	0.2892	0.0052	0.0866	0.6299	0.0043	0.1297	0.0010	0.0036
erb-2	0.7431	0.0011	0.1373	0.1373	0.0004	0.1557	0.1557	0.0057	0.0057	1.0000
c-ets 2	0.7527	0.0783	0.0782	0.0782	0.0244	0.0866	0.0866	0.0229	0.0229	1.0000
c-fos	0.6002	0.0258	0.0739	0.0113	0.0291	0.0495	0.0069	0.2604	0.0008	0.0023
c-jun	0.0308	0.0212	0.0329	0.0447	0.0767	0.7371	0.0579	0.0380	0.0008	0.1110
p38	0.3397	0.3397	0.3397	0.3397	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Mek 1	0.2188	0.0000	0.2188	0.2188	0.0122	1.0000	1.0000	0.0122	0.0122	1.0000
c-myc	0.2147	0.0063	0.2147	0.2147	0.1167	1.0000	1.0000	0.1167	0.1167	1.0000
NFkB	0.9735	0.9735	0.9735	0.9735	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
IkBa	0.0667	0.0038	0.0667	0.0667	0.0015	1.0000	1.0000	0.0015	0.0015	1.0000
PI 3Kp110b	0.1732	0.0000	0.1732	0.1732	0.0000	1.0000	1.0000	0.0000	0.0000	1.0000
p85	0.2451	0.0002	0.2451	0.2451	0.0281	1.0000	1.0000	0.0281	0.0281	1.0000
c-raf 1	0.0637	0.0001	0.0637	0.0637	0.0003	1.0000	1.0000	0.0003	0.0003	1.0000
GAP	0.3269	0.0103	0.3269	0.3269	0.0000	1.0000	1.0000	0.0000	0.0000	1.0000
c-src	0.5147	0.0021	0.5147	0.5147	0.0000	1.0000	1.0000	0.0000	0.0000	1.0000
E-cadherin	0.9442	0.0858	0.0116	0.0098	0.0239	0.0117	0.0098	0.0086	0.0084	0.1878
ICAM 1	0.0089	0.0010	0.0015	0.0793	0.0090	0.2563	0.0185	0.0305	0.0005	0.0085
NCAM 1	0.4397	0.3616	0.0503	0.0503	0.0330	0.0656	0.0656	0.0335	0.0335	1.0000
Integrin a 1	0.2404	0.0000	0.2404	0.2404	0.0000	1.0000	1.0000	0.0000	0.0000	1.0000
Integrin a2	0.1655	0.0002	0.0646	0.1655	0.0012	0.0688	1.0000	0.0015	0.0012	0.0688
Integrin a3	0.0285	0.0012	0.0004	0.0401	0.0140	0.0215	0.0116	0.2634	0.0009	0.0026
Integrin a4	0.9957	0.0000	0.1594	0.1594	0.0000	0.1701	0.1701	0.0000	0.0000	1.0000
Integrin a5	0.0617	0.0041	0.0617	0.0617	0.0007	1.0000	1.0000	0.0007	0.0007	1.0000
Integrin a6	0.2513	0.0317	0.9689	0.0218	0.0881	0.0888	0.0077	0.0243	0.0012	0.0197
Integrin aV	0.8266	0.0884	0.4932	0.8266	0.0453	0.3234	1.0000	0.0084	0.0453	0.3234
Integrin b1	0.7947	0.0296	0.0396	0.7947	0.0117	0.0160	1.0000	0.6545	0.0117	0.0160

Table II. *Continued*

	P(A:B)	P(A:C)	P(A:D)	P(A:E)	P(B:C)	P(B:D)	P(B:E)	P(C:D)	P(C:E)	P(D:E)
Integrin b3	0.2299	0.0000	0.2299	0.2299	0.0000	1.0000	1.0000	0.0000	0.0000	1.0000
Integrin b5	0.1923	0.0000	0.1923	0.1923	0.0001	1.0000	1.0000	0.0001	0.0001	1.0000
MUC 18	0.2451	0.0000	0.2451	0.2451	0.0001	1.0000	1.0000	0.0001	0.0001	1.0000
CD44	0.8057	0.0814	0.2529	0.0006	0.0688	0.2051	0.0006	0.2841	0.0002	0.0004
Angiopoietin 1	0.2408	0.2408	0.2408	0.2408	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Angiopoietin 2	0.2302	0.2302	0.2302	0.2302	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Endostatin	0.0007	0.0000	0.1332	0.1332	0.0000	0.0166	0.0166	0.0000	0.0000	1.0000
EGF	0.0294	0.0008	0.1093	0.1093	0.0060	0.0640	0.0640	0.0086	0.0086	1.0000
EGFR	0.0325	0.0126	0.0344	0.0344	0.3177	0.0101	0.0101	0.0055	0.0055	1.0000
FGF 2	0.1982	0.0048	0.0765	0.0765	0.0018	0.1340	0.1340	0.0190	0.0190	1.0000
FGFR2	0.8399	0.8399	0.8399	0.8399	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
VEGF	0.0347	0.0245	0.0069	0.0799	0.0011	0.1355	0.2847	0.0003	0.0018	0.0365
VEGFR	0.3193	0.0000	0.3193	0.3193	0.0001	1.0000	1.0000	0.0001	0.0001	1.0000
HGF	0.0420	0.0000	0.1624	0.1624	0.0000	0.0600	0.0600	0.0001	0.0001	1.0000
IFN alpha	0.1445	0.0000	0.1445	0.1445	0.0000	1.0000	1.0000	0.0000	0.0000	1.0000
IFN beta	0.7860	0.0000	0.1637	0.1637	0.0000	0.1805	0.1805	0.0001	0.0001	1.0000
IGF 1	0.2855	0.0008	0.2855	0.2855	0.0265	1.0000	1.0000	0.0265	0.0265	1.0000
IL 8	0.9045	0.0088	0.9045	0.9045	0.0051	1.0000	1.0000	0.0051	0.0051	1.0000
PDGF a	0.0507	0.0002	0.0507	0.0507	0.0002	1.0000	1.0000	0.0002	0.0002	1.0000
PDGF b	0.1856	0.0000	0.1856	0.1856	0.0000	1.0000	1.0000	0.0000	0.0000	1.0000
Tie 2	0.5589	0.4750	0.5589	0.5589	0.1040	1.0000	1.0000	0.1040	0.1040	1.0000
TGF b1	0.1491	0.1491	0.1491	0.1491	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
TGFBR1	0.0415	0.2664	0.0415	0.0415	0.0028	1.0000	1.0000	0.0028	0.0028	1.0000
Thrombospondin 1	0.0347	0.0028	0.0085	0.0278	0.0006	0.0014	0.9522	0.0076	0.0006	0.0010
Thrombospondin 2	0.4532	0.4532	0.4532	0.4532	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Kiss 1	0.0454	0.0001	0.0454	0.0454	0.0005	1.0000	1.0000	0.0005	0.0005	1.0000
Kai 1	0.0806	0.0012	0.0806	0.0806	0.0010	1.0000	1.0000	0.0010	0.0010	1.0000
MMP 1	0.0745	0.0015	0.0745	0.0745	0.0042	1.0000	1.0000	0.0042	0.0042	1.0000
MMP 2	0.1214	0.0000	0.1214	0.1214	0.0001	1.0000	1.0000	0.0001	0.0001	1.0000
MMP 9	0.1227	0.0000	0.1227	0.1227	0.0002	1.0000	1.0000	0.0002	0.0002	1.0000
Mta 1	0.2293	0.0000	0.2293	0.2041	0.0000	1.0000	0.9589	0.0000	0.0000	0.9589
Mts 1	0.0163	0.0044	0.0163	0.0140	0.0008	1.0000	0.9696	0.0008	0.0006	0.9696
Nm23-E4	0.0792	0.1940	0.0792	0.0792	0.0417	1.0000	1.0000	0.0417	0.0417	1.0000
uPA	0.8799	0.8799	0.8799	0.8799	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
uPAR	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Pai 1	0.0322	0.0020	0.0322	0.0322	0.0001	1.0000	1.0000	0.0001	0.0001	1.0000
Pai 2	0.0220	0.3653	0.0362	0.9027	0.0549	0.0007	0.0126	0.0007	0.1431	0.0070
Maspin	0.2359	0.2359	0.0487	0.2359	1.0000	0.0010	1.0000	0.0010	1.0000	0.0010
TIMP1	0.6948	0.0000	0.6948	0.6948	0.0000	1.0000	1.0000	0.0000	0.0000	1.0000
GAPDH	0.4229	0.0403	0.1026	0.1617	0.1426	0.3459	0.5081	0.5558	0.3736	0.7607
PPIA	0.9032	0.0780	0.0675	0.2369	0.0067	0.0078	0.0230	0.8668	0.2412	0.2052
Beta actin	0.1464	0.1427	0.1404	0.1464	0.6915	0.5132	0.9976	0.7752	0.6780	0.4951
No. of genes with $p<0.05$	16.0000	60.0000	15.0000	15.0000	58.0000	11.0000	13.0000	58.0000	62.0000	11.0000

reference was compared with pooled allogenic normal cells reference (Figure 2 B, E). The lowest, 11% of the tumour-related gene expression, was significant different ($p<0.05$) when pooled tumour reference was compared to either pooled allogenic normal cell (Table II D, B) or pooled patient normal squamous epithelial biopsies reference (Table II D, E). The highest, 62% of the tumour-related gene expression, was significantly different ($p<0.05$) when pooled cell lines reference was compared with pooled normal epithelial biopsies (Table II C, E).

Discussion

The identification of the genes involved in tumour response to therapy is crucial for understanding the underlying mechanisms of unfavourable outcome and the development

of individual treatment strategies. It has been reported that Bcl-2, Bad, TNF alpha, DR3, IGF1 and uPar play important roles in normal cells survival and malignancy (21-24). These genes were marginally altered in normal squamous epithelial cells and HNSCC biopsies in our investigation. The discrepancy between ours and previously reported results suggests specific functional genes in the given tissue types.

With cDNA array, the expression of large number of genes can be analysed in a single experiment. The commercial so-called "Universal reference RNA" obtained from a number of human-derived cell lines, pooled allogenic normal cells, pooled tumour type or even pooled normal matched tissue type, have been used as reference in the vast majority of cDNA array settings for clinically purpose. Pooled RNA from cell lines has been claimed to be the most suitable reference for the cDNA array approach (25).

The main purpose of using a reference is to obtain a common denominator for all experiments, making it possible to calculate a ratio and make comparisons between different experiments. The source of a reference is critical since each commercial agency and research group uses the pooled RNA from different cell lines or tissues types (2-9). Within the same group of tumour, differing gene expression profiles were detected when different sources of reference were used, as indicated in our investigation. The use of a non-specific reference might not provide a comparable picture of the gene profile of the given tumours.

It is well established that normal or tumour cells acquire genetic or epigenetic changes in order to survive as permanent growing cell lines *in vitro* (12, 14, 26). The up-regulation of various genes in pooled cell lines could be expected since they were immortalised and grew without any microenvironment limitation *in vitro*. The down-regulated VEGF and the unregulated 70% of the genes in pooled cell lines when compared with pooled normal cells supported our assumption.

The significant difference in gene expression between the normal cells of each individual and the pooled allogenic normal mixed cell types in our investigation suggested the tissue type-specific (27-29) and individual genetic background of each patient (30, 31). Within the pair of tumours and their autologous normal counterpart, a unique pattern of gene expression ratio was detected in each individual patient. Thus, not only the individual genetic background (31, 32) and tissue type specificity (27-29), but also the clinical stage of each tumour (6, 9, 30) need to be considered.

In conclusion, the selection of reference is critical for the evaluation of gene expression profiles obtained from cDNA array. Theoretically and our study indicated that the use of pooled human-derived cell lines, allogenic normal mixed cell types, tumour or even pooled allogenic normal matched tissue type as reference in cDNA array for identification of individual tumour-related gene expression will create only inconclusive and misleading information. The tumour gene expression profile with autologous matching tissue as reference will give the opportunity to compare across the samples, relative to each other and from various investigators. For clinical purposes, the patient autologous normal cells matching with the tumour type should be the most suitable reference in cDNA array analysis.

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