Electronic Supplementary Material (ESI) for Analytical Methods.

Supplementary Information

Systematic optimization and evaluation of sample pretreatment methods for LC-MS-based metabolomics analysis of adherent mammalian cancer cells

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The specific steps in each extraction protocol are detailed below.

In method E1, two 100% methanol extractions (500 μL each time) followed by an extraction with 250 μL of pure water were performed to extract metabolites. In method E2, a first extraction using 500 μL of acidic methanol (0.1% acetic acid) followed by an extraction with 500 μL of normal methanol and a final extraction with 500 μL of basic methanol (0.1% ammonium hydroxide) were performed. In method E3, cell pellets were resuspended in 1 mL of 100% methanol, vortexed at 3000 rpm for 3 minutes and incubated on ice for 10 minutes. In method E4, cell pellets were resuspended in 1 mL of ice-cold 50% aqueous acetonitrile, vortexed at 3000 rpm for 3 minutes and incubated on ice for 10 minutes. In method E5, cell pellets were resuspended in 1 mL of ice-cold 75% ethanol, vortexed at 3000 rpm for 3 minutes and incubated on ice for 10 minutes. In method E6, cell pellets were resuspended in 1 mL of 80% aqueous methanol, vortexed, incubated at 70 °C for 10 minutes, and then cooled on ice for 5 minutes. In method E7, cell pellets were resuspended in 1 mL of 75% aqueous ethanol, vortexed at 3000 rpm for 3 minutes, incubated at 70 °C for 5 minutes, and then cooled on ice for 5 minutes.

Table S1. Design of the optimization methods for each step.

Analyzed Content	Collection Batch	Quenching Time	Quenching Solvent	Cell Disruption Method	Extraction Solvent	Liquid Nitrogen Storage Time
1 Sample Collection	A20170112 A20170117 B20170112 B20170117	5 min	Q3	D2	E1	0 days
2 Quenching Time	same batch	0 min, 5 min, 10 min, 15 min, 20 min, 30 min	Q3	D2	E1	0 days
3 Quenching Solvent	same batch	5 min	Q1, Q2, Q3, Q4	D2	E1	0 days
4 Cell Disruption Method	same batch	5 min	Q3	D1, D2, D3	E1	0 days
5 Extraction Solvent	same batch	5 min	Q3	D2	E1, E2, E3, E4, E5, E6, E7	0 days
6 Sample Storage	same batch	5 min	Q3	D2	E1	0 days and 10 months

Table S2. Endogenous metabolites used to evaluate the different methods and method validation.

	No.	Compound	Formula	$[M+H]^+$ $[M-H]^-$	RT (min)	Instrument precision	Intra-day precision	Inter-day precision
Amino acids (19)	1	L-valine[a]	$C_5H_{11}NO_2$	118.0864	1.22	7.64%	5.40%	7.21%
	2	L-isoleucine[a]	$C_6H_{13}NO_2$	132.1018	1.88	5.13%	8.07%	11.26%
	3	L-leucine[a]	$C_6H_{13}NO_2$	132.1018	2.24	4.88%	4.50%	5.07%
	4	L-threonine[a]	$C_4H_9NO_3$	120.0658	1.09	7.72%	8.31%	10.92%
	5	L-tyrosine[a]	$C_9H_{11}NO_3$	182.0811	1.19	4.38%	8.93%	10.02%
	6	L-phenylalanine[a]	$C_9H_{11}NO_2$	166.0862	4.08	6.33%	4.31%	10.27%
	7	L-glutamine[a]	$C_5H_{10}N_2O_3$	147.0764	1.04	6.36%	10.67%	9.95%
	8	L-tryptophan[a]	$C_{11}H_{12}N_2O_2$	205.0972	5.00	4.28%	2.82%	3.97%
	9	L-proline[a]	$C_5H_9NO_2$	116.0706	1.23	3.08%	4.07%	10.48%
	10	L-glutamic acid[a]	$C_5H_9NO_4$	148.0603	1.21	5.11%	8.31%	12.02%
	11	L-aspartic acid[a]	$C_4H_7NO_4$	134.0447	1.21	8.21%	6.20%	6.53%
	12	L-arginine[a]	$C_6H_{14}N_4O_2$	175.1188	1.16	8.93%	16.15%	17.43%
	13	L-histidine[a]	$C_6H_9N_3O_2$	156.0766	1.08	8.49%	11.57%	11.72%
	14	Creatine[a]	$C_4H_9N_3O_2$	132.0768	1.24	7.80%	5.67%	5.94%
	15	L-ornithine[a]	$C_5H_{12}N_2O_2$	133.0973	0.94	8.22%	6.67%	11.94%
	16	L-methionine[a]	$C_5H_{11}NO_2S$	150.0583	1.19	9.04%	13.82%	18.20%
	17	L-serine[a]	$C_3H_7NO_3$	106.0499	1.13	8.32%	9.08%	8.51%
	18	Taurine[a]	$C_2H_7NO_3S$	126.0219	1.26	7.51%	9.89%	10.24%
	19	Dimethylarginine[a]	$C_8H_{18}N_4O_2$	203.1503	1.29	8.18%	8.03%	12.48%
Carnitines (10)	20	Carnitine C0[a]	$C_7H_{15}NO_3$	162.1123	1.19	9.68%	6.71%	7.88%
	21	Carnitine C2:0[a]	$C_9H_{17}NO_4$	204.1230	1.84	6.09%	7.34%	10.10%
	22	Carnitine C3:0[b]	$C_{10}H_{19}NO_4$	218.1387	3.54	9.92%	6.87%	13.02%
	23	Carnitine C4:0[b]	$C_{11}H_{21}NO_4$	232.1543	4.70	5.46%	8.04%	9.85%
	24	Carnitine C5:0[b]	$C_{12}H_{23}NO_4$	246.1700	5.58	9.22%	8.07%	8.39%
	25	Carnitine C6:0[b]	$C_{13}H_{25}NO_4$	260.1856	6.89	5.20%	9.56%	10.23%
	26	Carnitine C8:0[b]	$C_{15}H_{29}NO_4$	288.2169	8.72	5.99%	6.90%	7.07%
	27	Carnitine C10:0[b]	$C_{17}H_{33}NO_4$	316.2482	10.24	7.87%	7.32%	7.75%
	28	Carnitine C12:0[b]	$C_{19}H_{38}NO_4$	344.2795	11.98	8.57%	6.75%	12.45%
	29	Carnitine C14:1[b]	$C_{21}H_{39}NO_4$	370.2946	12.47	11.13%	11.69%	13.26%

Nucleotides (14)	30	Cytosine[a]	$C_4H_5N_3O$	112.0505	1.67	5.21%	7.86%	10.46%
	31	Uracil[a]	$C_4H_4N_2O_2$	113.0345	1.71	3.65%	6.29%	12.09%
	32	Hypoxanthine[a]	$C_5H_4N_4O$	137.0458	2.02	6.31%	6.51%	12.21%
	33	Xanthine[a]	$C_5H_4N_4O_2$	153.0407	1.69	6.00%	8.79%	12.80%
	34	Guanine[a]	$C_5H_5N_5O$	152.0567	1.72	7.83%	8.85%	12.71%
	35	1-Methylguanine[a]	$C_6H_7N_5O$	166.0723	2.16	7.56%	8.04%	9.60%
	36	Cytidine[a]	$C_9H_{13}N_3O_5$	244.0928	1.68	8.22%	12.00%	13.71%
	37	Uridine[a]	$C_9H_{12}N_2O_6$	245.0768	2.58	11.88%	9.28%	9.27%
	38	Adenosine[a]	$C_{10}H_{13}N_5O_4$	268.1040	3.41	5.95%	12.73%	14.15%
	39	Xanthosine[a]	$C_{10}H_{12}N_4O_6$	285.0830	3.70	11.36%	8.49%	9.83%
	40	Guanosine[a]	$C_{10}H_{13}N_5O_5$	284.0989	3.52	7.00%	8.57%	14.44%
	41	Succinyladenosine[b]	$C_{14}H_{17}N_5O_8$	384.1150	4.29	6.82%	5.85%	9.69%
	42	UMP[a]	$C_9H_{13}N_2O_9P$	325.0431	1.80	7.64%	5.54%	15.75%
	43	CMP[a]	$C_9H_{14}N_3O_8P$	324.0591	1.79	7.82%	8.60%	10.51%
Lysophosphatides (12)	44	LPC (14:0)[b]	$C_{22}H_{46}NO_7P$	468.3085	12.03	8.06%	14.00%	16.20%
	45	LPC (16:1)[b]	$C_{24}H_{48}NO_7P$	494.3241	12.71	6.06%	11.45%	12.50%
	46	LPC (16:0)[a]	$C_{24}H_{50}NO_7P$	496.3398	15.04	5.50%	9.49%	9.49%
	47	LPE (20:4)[b]	$C_{25}H_{44}NO_7P$	502.2928	13.69	5.51%	9.93%	10.36%
	48	LPC (17:1)[b]	$C_{25}H_{50}NO_7P$	508.3398	14.24	12.06%	9.33%	11.49%
	49	LPC (18:2)[b]	$C_{26}H_{50}NO_7P$	520.3398	13.73	7.10%	11.10%	12.99%
	50	LPC (18:1)[a]	$C_{26}H_{52}NO_7P$	522.3554	16.30	7.61%	12.78%	18.35%
	51	LPC (18:0)[b]	$C_{26}H_{54}NO_7P$	524.3711	15.73	10.93%	13.13%	13.65%
	52	LPE (22:6)[b]	$C_{27}H_{44}NO_7P$	526.2928	13.18	6.42%	5.87%	19.24%
	53	LPC (20:5)[b]	$C_{28}H_{48}NO_7P$	542.3241	12.45	8.80%	5.79%	5.74%
	54	LPC (20:4)[b]	$C_{28}H_{50}NO_7P$	544.3398	13.39	6.46%	6.69%	10.69%
	55	LPC (21:0)[b]	$C_{30}H_{50}NO_7P$	568.3398	13.76	9.61%	10.89%	11.05%
Organic acids (13)	56	Malonic acid[b]	$C_3H_4O_4$	103.0037	1.89	8.72%	9.29%	11.50%
	57	Succinic acid[a]	$C_4H_6O_4$	117.0193	2.30	9.88%	8.80%	13.07%
	58	Hippuric acid[a]	$C_9H_9NO_3$	178.0510	5.86	9.90%	8.62%	13.40%
	59	Pantothenic acid[a]	$C_9H_{17}NO_5$	218.1034	4.15	9.60%	13.22%	12.35%
	60	Citric acid[a]	$C_6H_8O_7$	191.0197	1.96	8.72%	9.93%	17.34%
	61	FAC 10:0-OH[b]	$C_{10}H_{20}O_3$	187.1340	10.84	8.47%	9.90%	13.65%

	62	FAC 12:1-OH[b]	$C_{12}H_{22}O_3$	213.1496	11.42	8.42%	9.89%	9.88%
	63	FAC 12:0-OH[b]	$C_{12}H_{24}O_3$	215.1653	11.00	5.39%	8.53%	8.39%
	64	FAC 14:1-OH[b]	$C_{14}H_{26}O_3$	241.1809	14.84	9.08%	9.70%	11.11%
	65	FAC 16:0[b]	$C_{16}H_{32}O_2$	255.2330	23.54	9.51%	8.42%	11.25%
	66	FAC 18:1[b]	$C_{18}H_{34}O_2$	281.2486	23.74	5.56%	9.54%	10.90%
	67	FAC 18:0[b]	$C_{18}H_{36}O_2$	283.2643	24.84	9.20%	10.27%	18.54%
	68	FAC 20:4[a]	$C_{20}H_{32}O_2$	303.2330	22.77	8.68%	6.57%	9.84%

[a] Metabolites confirmed using standard compounds. [b] Metabolites identified by database searches and MS fragmentation. The data of amino acids, carnitines, mucleotides and lysophosphatides was acquired in ESI positive ion mode, and the data of organic acids was acquired in ESI negative ion mode.

Table S3 Comparison of mean normalized values of metabolites processed using different quenching solvents (Related to Fig. 3D).

Classify	Q1		Q2		Q3		Q4	
	mean	CV %	mean	CV %	mean	CV %	mean	CV %
Amino acids	0.98	10.48	0.57	9.32	0.87	8.45	0.73	7.61
Carnitines	0.30	9.49	0.32	15.21	0.31	13.19	0.41	14.48
Nucleotides	0.49	14.01	0.53	34.20	0.56	15.25	0.58	13.71
Lysophophatides	0.56	12.47	0.55	13.20	0.72	15.94	0.17	16.81
Organic acids	0.40	11.48	0.37	17.82	0.44	11.97	0.45	17.16

Table S4 Comparison of mean normalized values of metabolites using different extraction protocols (Related to Fig. 5A).

Classify	E1		E2		E3		E4		E5		E6		E7	
	mean	CV %	mean	CV %	mean	CV %	mean	CV %	mean	CV %	mean	CV %	mean	CV %
Amino acids	0.52	8.93	0.27	24.48	0.23	12.06	0.29	6.09	0.27	6.88	0.31	5.37	0.27	7.50
Carnitines	0.14	11.67	0.10	20.17	0.15	3.83	0.09	3.49	0.16	12.95	0.09	7.38	0.10	12.38
Nucleotides	0.20	9.49	0.15	6.90	0.07	3.63	0.58	8.05	0.12	11.74	0.07	15.93	0.18	6.75
Lysophophatides	0.37	8.34	0.24	20.68	0.51	12.77	0.31	10.89	0.33	14.03	0.33	16.13	0.29	5.09
Organic acids	0.31	8.05	0.11	22.42	0.13	10.91	0.15	16.68	0.18	14.07	0.22	13.01	0.24	14.76

Table S5 Comparison of mean normalized values of nucleotides, nucleosides and nucleobases using different extraction protocols (Related to Fig. 5B).

Classify	E1		E2		E3		E4		E5		E6		E7	
	mean	CV %	mean	CV %	mean	CV %	mean	CV %	mean	CV %	mean	CV %	mean	CV %
Guanosine	0.02	5.03	0.00	6.71	0.00	7.19	0.14	5.55	0.01	6.19	0.01	18.94	0.01	8.45
Adenosine	0.03	8.93	0.00	15.82	0.00	6.80	0.14	11.59	0.00	12.14	0.01	29.58	0.01	8.79
Succinyladenosine	0.01	4.86	0.00	16.94	0.00	8.82	0.13	6.23	0.01	12.65	0.01	22.97	0.01	8.39
Uridine	0.01	5.94	0.00	8.80	0.00	3.62	0.15	18.26	0.00	17.75	0.00	16.60	0.00	8.53
Xanthosine	0.02	11.50	0.01	8.64	0.01	29.92	0.10	10.64	0.02	12.10	0.01	32.99	0.02	11.98
Cytosine	0.04	3.88	0.03	20.07	0.01	2.38	0.05	2.85	0.02	6.07	0.01	15.07	0.04	9.05
Cytidine	0.04	4.44	0.03	20.29	0.01	4.57	0.05	4.93	0.02	8.02	0.01	13.05	0.04	8.84
Uracil	0.05	38.97	0.03	50.85	0.01	1.36	0.05	19.48	0.03	54.57	0.01	5.58	0.03	7.66
Guanine	0.04	3.07	0.04	18.39	0.01	2.13	0.04	8.11	0.02	3.06	0.02	34.95	0.04	7.14
Hypoxanthine	0.03	14.75	0.03	11.76	0.01	27.11	0.04	18.36	0.02	20.53	0.03	18.10	0.04	15.00
UMP	0.04	4.82	0.04	31.12	0.03	5.04	0.00	8.33	0.04	9.16	0.01	13.91	0.04	8.79
CMP	0.03	18.87	0.04	45.44	0.03	9.05	0.02	8.30	0.03	10.45	0.02	15.33	0.03	13.68

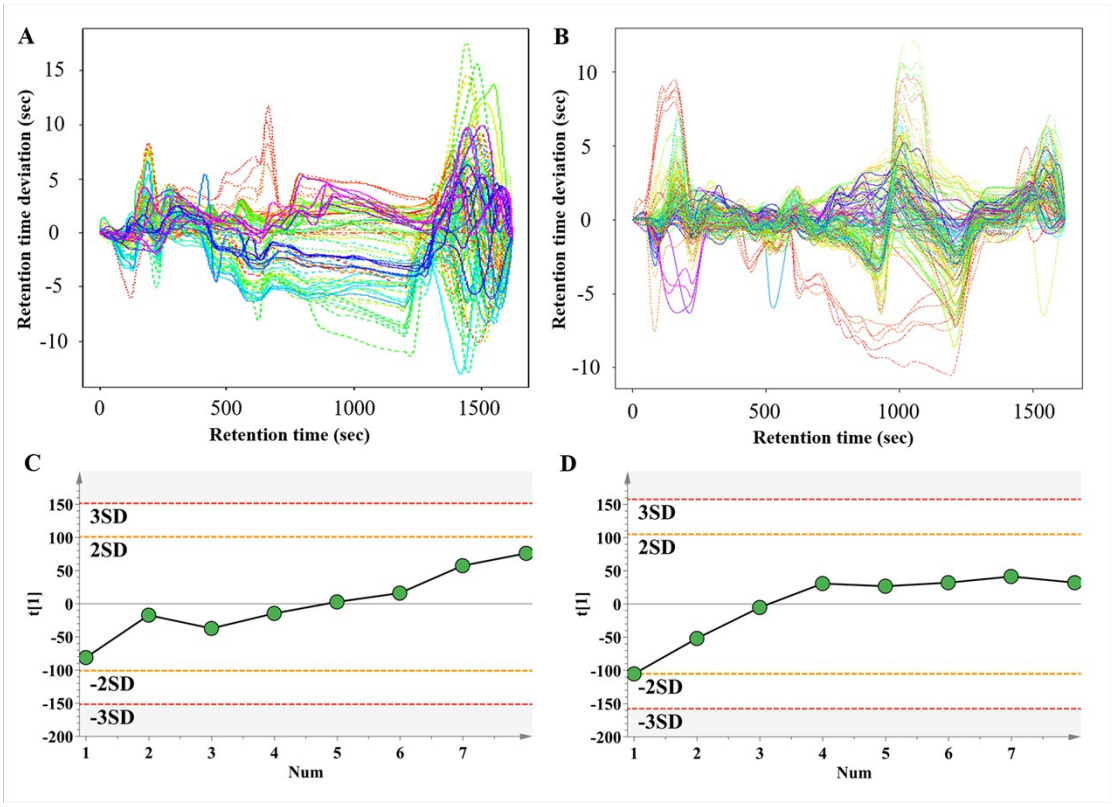

Fig. S1 Retention time deviation plots of all samples from LC-(+) ESI-MS data (A) and LC-(-) ESI-MS data (B), and PCA score plots of all QC samples from LC-(+) ESI-MS data (C) and LC-(-) ESI-MS data (D).

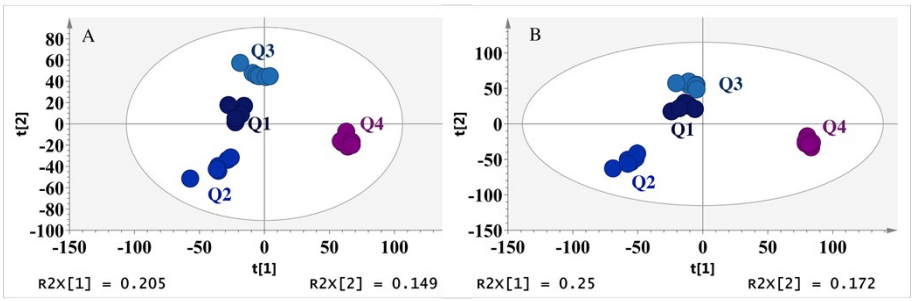

Fig. S2 PCA score plots based on the LC–(+) ESI–MS (A) and LC–(-) ESI–MS (B) data sets for different quenching solvents

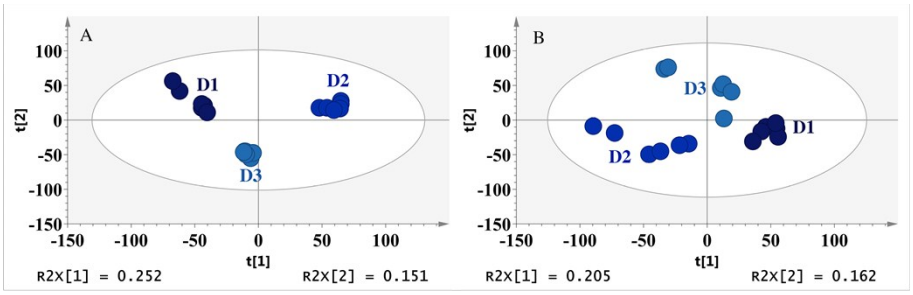

Fig. S3 PCA score plots based on the LC–(+) ESI–MS (A) and LC–(-) ESI–MS (B) data sets for different disruption methods.

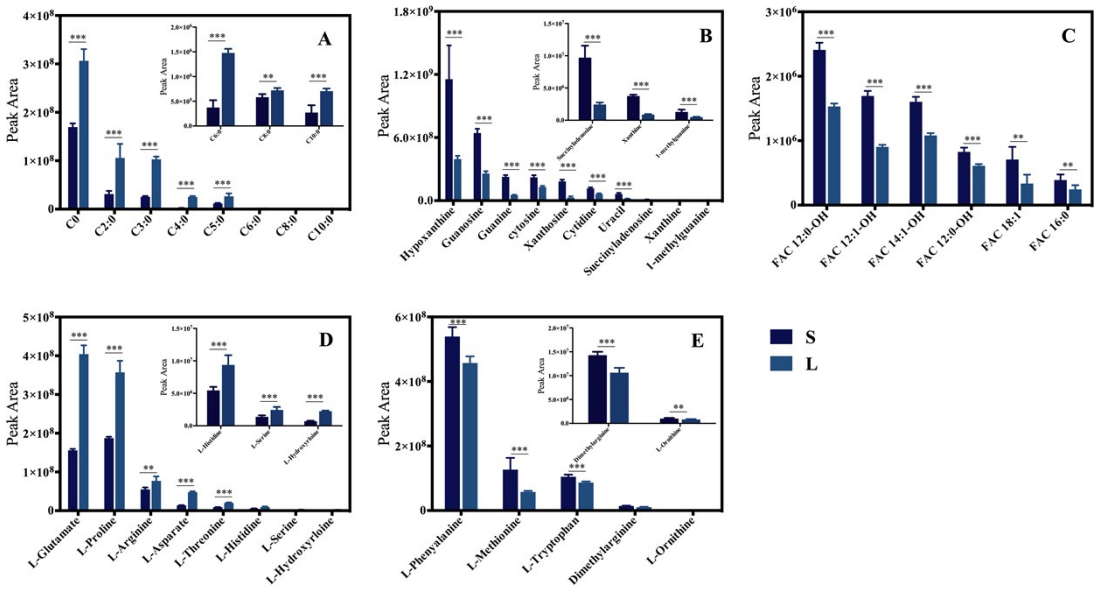

Fig. S4 Comparison of cell samples frozen in liquid nitrogen for 0 days and 10 months (related to Fig.7). (A) Increased carnitines after long-term storage. (B) Decreased nucleosides and nucleobases after long-term storage. (C) Decreased fatty acids after long-term storage. (D) Increased amino acids after long-term storage (E) Decreased amino acids after long-term storage. (n = 6) (A, B, D and E: the insets show an expanded Y-axis for the lower-intensity metabolites in the main panels) L: long-term-storage cell samples (frozen for 10 months in liquid nitrogen). S: immediately processed cell samples (frozen for 0 days in liquid nitrogen). *** denotes $p < 0.001$; ** denotes $p < 0.01$; * denotes $p < 0.05$. Error bars represent standard deviations.

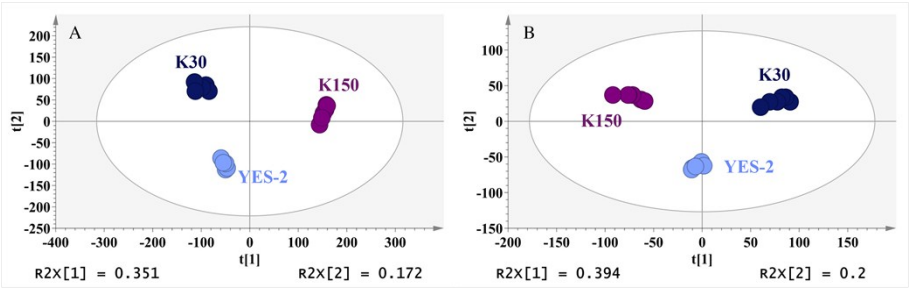

Fig. S5 PCA score plots based on the LC–(+) ESI–MS (A) and LC–(-) ESI–MS (B) data sets for three human esophageal squamous cell carcinoma cell lines