Effects of Cigarette Smoke Extract on Skin Ageing

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Introduction: Nature versus Nurture. This long standing debate permeates its way through all fields of study from philosophy to the physical and social sciences. The condition of our skin can also be affected by both genetic and epigenetic factors. Our genes could dictate whether we develop freckles on our face and perhaps the natural colour of our hair. Epigenetic factors (our environment and habits) like spending time in the sun or smoking cigarettes could determine how tan our skin gets or increase the likelihood that one develops cancer. Cigarette smoke components that enter our bodies have been shown to induce Matrix Metalloproteinases(MMP) 1 and 3 mRNA in skin fibroblasts that degrade and age skin (Freyn-3 Apr 2018)[1]. Smoking also appears to increase the risk of developing cancer(Lahmann-Mar 2001)[2] As a way to convince my father to stop smoking, it is of interest to me to demonstrate the effect cigarette smoke extract (CSE) would have on the skin, particularly dermal fibroblasts that support it. This investigation aims to observe what effect cigarette smoke extract will have on the transcription of 5 genes related to skin proteins and cancer in healthy fibroblasts. The 5 primer pairs will be used to amplify fibroblast cDNA by Polymerase Chain Reaction(PCR) and analysed through gel electrophoresis: COL1 collagen gene, GAPDH housekeeping gene, HRAS proto oncogene, TP53 tumour suppressor gene and ACTA2 actin producing gene. Bisulfite converted (BC) sequences of control and perturbed ELOVL2 and COL1 will also be analysed for their methylation status. . This leads us to hypothesise the upregulation of ELOVL2 & HRAS; and downregulation of TP53, ACTA2 and COL1.

Method: Dermal Fibroblasts cells were thawed from a cryovial and cultured. The batch was split into 2, Perturbation(perturbed with CSE) and the Control. The cells were lysed to harvest their RNA for cDNA synthesis and amplified by PCR with the primers we were given and designed. Gel electrophoresis helped determine our PCR products.
Day 1: A cryovial of Fibroblasts was thawed in a 37^{o}C water bath and then suspended in 1ml DMEM in a 15ml centrifuge tube, centrifuged (1400rpm/300G for 4 minutes(min)), and resuspended in 5ml DMEM. Cells were transferred to a T25 flask to grow in an incubator (37^{o}C, 5% CO_2) for 2 days before passaging the cells.
Day 3: The cells were visualised under a microscope to check for survival, washed with Phosphate Buffered Saline(PBS) solution and Trypsinised(37^{o}C for 5 min) to detach the cells from the flask before inhibiting the reaction with 5ml of DMEM. The cell solution was transferred to a 15ml centrifuge tube and centrifuged. The supernatant was discarded and the cell pellet resuspended in 5ml DMEM to remove Trypsin that is toxic to fibroblasts. Cells were counted by placing a 10uL aliquot in a haemocytometer. Two 6-well plates were labelled (Control and CSE Perturbation). The cell solution was made up to 12ml with DMEM and distributed evenly to 3 wells on each well plate (6 wells total) and criss-crossed to ensure the bottom was completely covered. Well plates were left to incubate (37^{o}C, 5% CO_2) for 5 days before perturbation.
Day 8: The media on all well plates were discarded. The Control plate had 2ml fresh DMEM added to each well with cells. The CSE Perturbation plate had 2ml CSE media added in each well instead. The cells were incubated (37^{o}C, 5% CO_2) for 2 days before RNA harvesting.
Day 10: The media from all wells were discarded, washed with PBS, then discarded. 350uL of RNAeasy Lysis Buffer(RLT) was added to a populated well of the Control plate, scraped with a cell scraper to detach cells from the plate, transferred to the next well to repeat the steps until all cells of the well plate were in solution, then pipetted to a 1.5ml centrifuge tube.

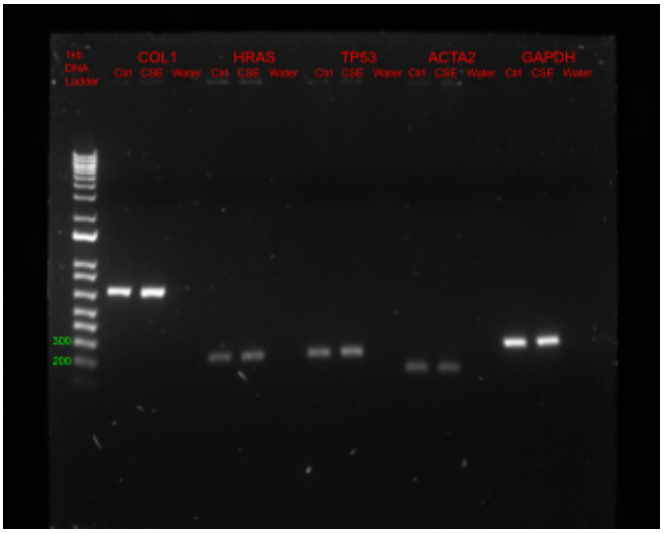
Steps repeated separately for the CSE Perturbation plate. The samples were lysed for RNA isolation using the Qiagen Mini Plus Kit and Qiashredder. The RNA was eluted with 30uL of RNase-free water, then quantified by nanodrop and stored(-80ºC) before cDNA synthesis and PCR.

Day 15: cDNA synthesis was done with the SuperScript IV kit. 200ng of sample RNA, water to total 11uL and 2uL of a stock solution(OligodT, dNTPmM, Template RNA) was added to a PCR tube for both samples. Both tubes were heated in a PCR machine(65ºC, 5 min) and cooled on ice. A reverse transcriptase reaction mix(SSIV buffer, DTT, RNase inhibitor, Reverse Transcriptase) was added to both tubes and heated in a PCR machine(50ºC, 10 min then 80ºC, 10 min). For PCR, 15 tubes were labelled(3 per gene to analyse: Control, Perturbed, Water) and filled with 1uL of Control/Perturbed/Water sample and 29uL of a PCR master mix(Master Mix, DMSO, water, 10uM of Forward/Reverse primer mix). All tubes underwent PCR cycle: 95ºC for 2 min, 35 cycles of 95ºC for 20sec, 50-62ºC for 20 sec, 72ºC for 30 sec, then 72ºC for 5min before ending at 4ºC.

Day 22: Gel block with wells was prepared by pouring a heated agarose mix into a prepared gel box with a 16 pronged comb and left to cool and solidify. Gel box with gel was moved to gel tank, submerged in TAE buffer and had the comb removed. The first well was loaded with a dyed DNA ladder and the other 15 samples were dyed and loaded into the remaining wells. Gel electrophoresis ran at 180V for 30min before imaging.

Primers 5'-3'	COL1	HRAS	TP53	ACTA2	GAPDH
Forward Primer	ATCACCTGCG TACAGAACGG	TGGTCATTGATG GGGAGACG	CCGAGTGGAAG GAAATTTGC	GAAAATGAGAT GGCCACTGC	CGTCTTCACC ACCATGGAGA
Reverse Primer	CTGTGTCCCT TCATTCCAGG	AGCCAGGTCAC ACTTGTTCC	ACAAACACGCA CCTCAAAGC	GTAGGTGGTTT CATGGATGC	CGGCCATCAC GCCACAGTTT

Results: BC sequences were used to analyse the methylation status of COL1 and ELOVL2 genes using CodonCode Aligner software, when perturbed with CSE. Each gene had a control and perturbed sequence to compare. The sequences were first trimmed to remove all low-quality bits in the data, then assembled to determine the likely sequence ("contig") we get from the various samples before isolating our target amplicon with forward and reverse primers. Overall CSE perturbation appears to increase methylation of certain CpG sites on both COL1 and ELOVL2 amplicons.



	A	B	C	D	E	F	G	H	I	J	K	L
1	COL1											
2	CG/TG Site No.	1	2	3	4	5	6	7	8	9	10	11
3	Control Methylation Count (9 Samples)	1	1	0	1	0	0	0	0	0	1	9
4	CSE Methylation Count (5 Samples)	0	0	0	0	0	0	4	4	3	0	5
5	Control Methylation%	11	11	0	11	0	0	0	0	0	11	100
6	CSE Methylation %	0	0	0	0	0	0	80	80	60	0	100
7	Overall Methylation % (Control) =	13 %										
8	Overall Methylation % (CSE) =	29 %										
9												
10	ELOVL2											
11	CG/TG Site No.	1	2	3	4	5	6	7	8	9		
12	Control Methylation Count (8 Samples)	0	0	0	2	2	0	2	0	0		
13	CSE Methylation Count (10 Samples)	1	1	1	3	2	1	1	0	0		
14	Control Methylation%	0	0	0	25	25	0	25	0	0		
15	CSE Methylation %	10	10	10	30	20	10	10	0	0		
16	Overall Methylation % (Control) =	8 %										
17	Overall Methylation % (CSE) =	11 %										

Figure 1: (Left)Gel Electrophoresis Image, Columns Left to Right: 1kb DNA Ladder; COL1 Control, Perturbed, Water; HRAS Control, Perturbed, Water; TP53 Control, Perturbed, Water; ACTA2 Control,Perturbed, Water; GAPDH Control, Perturbed, Water. (Right)Table showing CpG sites found after Bisulfite Conversion(BC) sequencing and comparing to the original sequence.

Control and Perturbed Bisulfite Converted(BC) Sequences for COL1 and ELOVL2 were scanned for CpG sites, using the original sequence as a reference. COL1 had 11 CpG sites while for ELOVL2, we were asked to focus on 9 specific CpGs. The Percentage(%) Methylation was calculated for each CpG site, Control and Perturbed, by dividing the number of CGs by the number of samples at the site and multiplying by 100. The Overall Methylation Percentage took an average of these percentages. COL1 Methylation increased from 13% to 29% and ELOVL2 Methylation increased from 8% to 11%. This suggests that CSE perturbation likely caused hypermethylation in these regions and perhaps downregulation of these genes if the same pattern applies to the entire gene sequence. In fibroblast culture, we might then expect less collagen and fatty acid production in the CSE perturbed culture than the control.

Gel electrophoresis helped to determine the concentration of amplicon products for each of the primer sets (COL1, HRAS, TP53, ACTA2, GAPDH) for both control and perturbed PCR products. The intensity of each band is likely proportional to the concentration of DNA present. The absence of bands on all “Water” columns indicate that there was likely no other DNA contamination in our samples. To determine small differences in intensity, a pixel RGB value tool was used to determine the intensity of each band. Higher values imply greater band intensity. We observe that the control and perturbed COL1 and GAPDH bands are similar in intensity. The perturbed HRAS and TP53 bands were very slightly brighter than their controls while the perturbed ACTA2 band was slightly less intense than its control. This suggests that COL1 and GAPDH do not appear to show significant changes in transcription, however considering that their bands are very bright, it would be difficult to tell minute differences. HRAS and TP53 both seem to be upregulated after being perturbed with CSE while ACTA2 was downregulated. HRAS, TP53 and ACTA2, the genes whose primers we designed, came out between the 200-300 bp bands as designed, using the DNA ladder on the left for reference.

Discussion: Each BC set(control/perturbed) had a varying number of samples ranging from 5-10. This inconsistency might have made the strand comparison less reliable in terms of the methylation percentages. Perhaps having 10 or more samples for each set of sequences would make the comparison more reliable. COL1 did not appear to be up or downregulated much in the gel image since the band intensity was visually similar between the control and perturbed bands. The BC sequences, however, helped to determine the actual COL1 methylation status, which was hypermethylated after perturbation. This suggests some downregulation of COL1. Perhaps this might’ve been more prominent in the gel image if the saturation was not too high. The BC sequencing also suggests perturbed ELOVL2 was hypermethylated and thus downregulated. This could result in reduced lipid synthesis and thus ageing phenotypes since ELOVL2 tends to be a biomarker for age(Li-25 May 2022)[3]. GAPDH is a housekeeping gene. Its expression tends to be consistent through a large variety of experiment conditions and was used as a control for gel electrophoresis to ensure that gel electrophoresis ran properly(Zainuddin-14 Aug 2010)[4]. The similarity in band brightness between the control and perturbed GAPDH indicate that a similar amount of nucleic acids were loaded into each well for the other genes as well. The similar band distance suggests that electrophoresis ran well. HRAS proto oncogene and TP53 tumour suppressor genes both appear to be upregulated after CSE perturbation. Michalska et al observed increased HRAS mRNA in the blood of smokers, suggesting that upregulation of HRAS expression is consistent with this study (Pązik- 6 Feb 2021)[5]. Contrary to our hypothesis that CSE would downregulate TP53 tumour suppressor gene, its upregulation is actually consistent with a study by Pezzuto et al. They claimed that CSE downregulated RNA-binding motif protein 5(RBM5) which in turn upregulated TP53 expression (Pezzuto-3 May 2019)[6]. ACTA2 was downregulated as per our hypothesis and is consistent with a similar study by Obernolte et al (Obernolte-Feb 2022)[7].
Overall, upregulated HRAS likely suggests increased cell division and MMP production. Contrarily, upregulated TP53 suggests reduced cell division and cell senescence.

Downregulated ACTA2 and COL1 from bisulfite conversion suggests reduced actin and collagen production respectively. The combined effects of increased MMP production, cell senescence and reduced actin and collagen production all contribute to the degradation and ageing of skin. Thus our results can be interpreted with support of other studies that CSE likely does induce skin ageing and could potentially be carcinogenic. Our hypothesis holds except for the TP53 gene where it was upregulated instead. Yet this upregulation can indeed contribute to skin ageing. Opposing effects such as increased and decreased cell division from HRAS and TP53 upregulation respectively could cancel out on the population level as shown from gel electrophoresis. On a cellular level however, excessive upregulation of one of these genes could cause cell senescence and ageing or cancer.
In conclusion, we hypothesised upregulation of ELOVL2 & HRAS; and downregulation of TP53, ACTA2 and COL1. We tested this by first culturing unperturbed(control) and CSE perturbed skin fibroblasts. We extracted their RNA to undergo cDNA synthesis using Reverse Transcriptase then amplified the specific gene sequences using primers we designed by PCR. The products went through Gel Electrophoresis and imaged to be analysed. Our results show that our hypothesis is correct except for TP53 which was upregulated, and still contributes to skin ageing. The results of this study show that CSE does induce skin ageing by degrading skin fibroblasts and dermal proteins but could potentially cause cancer. Our results are summarised in the table below.

	COL1	HRAS	TP53	ACTA2	GADPH	ELOVL2
BC Sequencing	Hypermethylated	NIL	NIL	NIL	NIL	Hypermethylated
Gel Electrophoresis	No change	Upregulated	Upregulated	Downregulated	No Change	NIL
Overall	Downregulated	Upregulated	Upregulated	Downregulated	No Change	Downregulated
Ageing effect	Less Collagen Produced	More MMPs break down ECM	More Cell Senescence	Less Actin Produced	NIL	Less fatty acids produced

Figure 2: Summary Table of results due to CSE perturbation.

References:

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