The Involvement of Tsp-9 and CELE_K02E10.4 on Chemoperception in C. *elegans* Using Chemotaxis Index Analysis

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Abstract

The expression of specific genes is critical in displaying proper sensory reception of the environment. Hence, the knockdown of such genes can cause debilitating outcomes in chemoreception. This study aimed to investigate the involvement of genes tsp-9 and CELE_K02E10.4 on chemoperception in C. *elegans* using a chemotaxis index analysis. With tsp-9 and CELE_K02E10.4 being hypothesized to be involved in chemoreception, the genes were knocked down using RNAi. Worms were incubated with bacteria containing C. *elegans* genes, allowing progeny to be exposed to RNAi, and a chemotaxis index using C. *elegans'* migration was calculated. That is, C. *elegans* were said to migrate toward either the diacetyl side (a chemical attractant) or the sodium azide side (an anesthetic). The data was visualized and compiled into block-reported and course-reported datasets. After Z-statistics, it was determined the genes tsp-9 and CELE_K02E10.4 do not have a significant impact on C. *elegans* chemotaxis abilities due to the P-values, excluding the positive control (L440 gene), being greater than 0.05. The mean chemotaxis index for both datasets of the genes strayed from 0.5 which, if seen, would indicate the genes affect chemotaxis ability due to random dispersion of worms and inability to sense diacetyl. This result was not found. Instead, previous studies point to tsp-9 not being involved in chemoreception, rather, in immunology, with no major studies on the CELE_K02E10.4 gene's biological significance. It was instead inferred CELE_K02E10.4 is not involved in chemoreception due to the gene's placement in the membrane. In conclusion, the initial hypothesis that tsp-9 and CELE_K02E10.4 are involved in chemoreception was refuted and one fails to reject the null hypothesis. However, the sparse studies on tsp-9 and CELE_K02E10.4 provide space for future endeavors of said genes within molecular biology, upholding the importance of chemoreception of environmental stimuli.

Introduction

The ability of an organism to effectively navigate and sense its environment is pivotal in its survival and reproducibility. Chemoreception of environmental stimuli, for example, allows an organism to move in response to chemical gradients and translate environmental cues into amplified intracellular signaling. These signals lead to elongated cell shape, actin polymerization, and movement along chemical gradients (Wang et al., 2011). Chemoreception is a major sensory system fundamental to various life processes involving nutrient targeting and damage avoidance in harmful environments. The study of sensory reception and the cellular navigation process is thus vital in understanding how a myriad of organisms interact with the external environment. The omnipresent integration of chemotaxis in the function of diverse organisms, including humans, furthers the importance of understanding the mechanisms of chemoreception. In humans, chemotaxis is essential for a multitude of physiological circumstances, such as wound healing, axon guidance, and tissue development (Wang et al., 2011). Due to chemoreception being mediated by complex gene families (Robertson et al., 2006), the process can be investigated using RNA biology. RNA plays a tremendous role in the regulation of gene expression. Similarly, RNA interference (RNAi) involves small RNAs that regulate the expression or translation of targeted genes in a multitude of eukaryotic organisms (Kim D.H., Rossi J.J., 2008). Due to the suited function of RNAi, RNAi can be used to knock down the expression of proteins likely involved in sensing and responding to chemical signals. This makes RNAi a well-suited method to investigate the genes involved in chemotaxis.

This study aimed to discover the expression of genes integral to chemotaxis, using Caenorhabditis elegans as a model organism. It is known that the chemoreceptor Odr-10 is necessary for response to chemoattractants in C. *elegans* due to the removal of the protein being involved with abnormal nervous system function in chemotaxis (Department of Molecular, Cellular, and Developmental Biology UCSB, 2023). In this study, two more genes were investigated using RNAi: tsp-9 and CELE_K02E10.4. Tsp-9 is a protein-coding gene with human ortholog(s) of the gene involved in immunodeficiency 6. CELE_K02E10.4 is a protein-coding gene predicted to be located in the membrane. (The Alliance of Genome Resources, 2023). The multitude of unknowns involving these genes underscores their importance in studying them. Additionally, potentially functional chemoreceptor genes and pseudogenes make up 7% of the gene complement of C. *elegans*, more than any other sequenced genome (Robertson et al., 2006). Thus, it is likely that tsp-9 and CELE_K02E10.4 are involved in chemoreception and will impair chemotaxis once knocked down using RNAi, further making these genes important to study. If genes tsp-9 and CELE_K02E10.4 are involved in chemoattraction, then the chemotaxis index for organisms with a treatment affecting tsp-9 and CELE_K02E10.4 should be approximately 0.5 because there would be a negative effect on the neurosensory pathway for attractants, causing worms to be randomly distributed. The hypothesis was addressed by observing L4 C. *elegans* on an agar plate over a two-week period.

Methodology

To conduct the investigation, RNAi plates were prepared to knock down genes predicted to be involved in chemoattraction, using Caenorhabditis elegans as a model organism. The advantages of using C. *elegans* as a model are due to its short life cycle, compact genome, anatomical simplicity, simple propagation, small size, and transparency. The RNAi plates were prepared by adding 3-5 worms to four different RNAi plates containing bacteria transformed with C. *elegans* genes. An L4440 negative control, ODR-10 positive control, and two randomly assigned experimental plates containing either tsp-9 or CELE_K02E10.4 were mixed onto separate plates and labeled. Tsp-9 and CELE_K02E10.4 had unknown effects on chemotaxis and chemo sensation prior to experimentation. The C. *elegans* in the L4 stage were transferred to the RNAi plates by mixing the tube containing the L4 worms in M9 solution, pipetting out 8 μL from the center of the tube, and dispensing the worms at the outer ring of the bacterial lawn on the RNAi plate. A dissecting microscope was used to determine the quantity of L4 worms added to the RNAi plates, ensuring crowding of worms did not occur. If crowding occurred, the worms would run out of food (bacteria containing C. *elegans* genes) harming their ability to propagate in the presence of the bacteria which was necessary for the chemotaxis assay. The RNAi plates were Para filmed and incubated for a week. Incubation promoted the C. *elegans* to produce progeny exposed to RNAi, which allowed one to test if altered chemoreception had occurred using a chemotaxis assay.

A chemotaxis assay was executed for each RNAi plate that was incubated. A chemotaxis plate with sodium azide – an anesthetic – and diacetyl (DA) — a chemical attractant — were deposited on opposite ends of the plate, and RNAi-propagated C. *elegans* were introduced at the center. To transfer the worms to the center of the plate, a filter-screen method was used after swirling the plate with a small quantity of water; wearing gloves, a Kimwipe was positioned under the filter, one pipetted to remove worms from the liquid of the RNAi plate, and the worms were transferred dropwise onto the filter screen. The worms were washed on the filter with additional nano pure when harvested. The screen was inverted at the center of the chemotaxis plate and gentle pressure was applied to facilitate the worms onto the agar. This was done for each of the four RNAi conditions.

After allotting the C. *elegans* 60 minutes of migration time, the number of worms on each side of the four plates was counted using the dissecting microscope, selected for its expansive field of view. The chemotaxis plate was centered on a transparent grid where the time of addition and time for scoring was also recorded. The Chemotaxis index (P) was then calculated using Equation 1 below where A and B represent the number of worms on the DA side and the number of worms on the sodium azide side, respectively. Data was analyzed using Z-statistics given in Equation 2 below. $\hat{p}1$, $\hat{p}2$, n_1, and n_2 are the mean values for the chemotaxis index of the experimental and control groups, and the sample sizes for experimental and negative control groups, respectively. A Z-test was performed to provide confidence in rejecting or failing to reject the hypothesis and was used to compare the experimental treatment to the negative control. It was determined whether the two variables were statistically different and not due to random procedural deviation. If the Z-score is less than or equal to 0.05 the data is statistically significant, and one can reject the null hypothesis.

Equation 1:

$$\text{Chemotaxis Index } (P); P = \frac{A}{(A+B)}$$

Equation 2:

$$Z = \frac{(\hat{p}_1 - \hat{p}_2)}{\sqrt{\frac{\hat{p}_1(1-\hat{p}_1)}{n_1} + \frac{\hat{p}_2(1-\hat{p}_2)}{n_2}}}$$

Results

Following data collection, it was shown that the genes Tsp-9 and CELE_K02E10.4 do not have a significant impact on C. *elegans* chemotaxis abilities. Chemotaxis index (CI) data generated from block- and course-level investigation were analyzed using Z-statistics seen in Equation 2, and an associated P-value was generated. As depicted in Table 1, the average CI for the block-level investigation for Tsp-9 and CELE_K02E10.4 was 0.680 and 0.670, respectively. Under Tsp-9 block data, Table 1 reports a Z-score of -0.625 with a P-value of 0.264. Under CELE_K02E10.4 block data, Table 1 reports a Z-score of -0.336 with a P-value of 0.367. The P-values for both experimental genes are greater than 0.05, signifying insignificant data – one fails to reject the null. The same result is seen with course-collected data. Table 1 shows the average CI for the course-level investigation for Tsp-9 and CELE_K02E10.4 was 0.700 and 0.730, respectively. The course-level Z-score and P-values for Tsp-9 and CELE_K02E10.4 were -0.594, 0.278, and -0.311, 0.378, respectively. Both P-values were greater than 0.05 denoting insignificant data and one fails to reject the null. Tsp-9 and CELE_K02E10.4 do not significantly affect chemoattraction.

Significant data was found examining the positive control, ODR-10. Table 1 shows the mean CI for the block- and course-reported data of ODR-10 was 0.478 and 0.565, respectively. The ODR-10 Z-score and P-values for block and course-reported data were 1-.72, 0.0427, and -3.86, 0.0001, respectively. Both P-values were less than 0.05 denoting significant data. ODR-10 significantly affects chemoattraction.

Table 1

Treatment	Level	Sample Size	Average CI	St. Dev.	Z-Score	P-Value
Positive Control (ODR-10)	Block	15	0.478	0.210	-1.72	0.0427
Negative Control (L440)	Block	16	0.765	0.120		
Tsp-9	Block	14	0.680	0.207	-0.625	0.264
CELE_K02E10.4	Block	3	0.670	0.163	-0.336	0.367
Positive Control (ODR-10)	Course	182	0.565	0.172	-3.86	0.0001
Negative Control (L440)	Course	178	0.754	0.158		
Tsp-9	Course	26	0.700	0.207	-0.594	0.278
CELE_K02E10.4	Course	34	0.730	0.187	-0.311	0.378

Table 1: Table of the average Chemotaxis Index with two subsets of data with Z-score. ODR-10 gene was knocked down for the positive control. L440 gene was knocked down for the negative control. Tsp-9 and CELE_K02E10.4 were experimental genes that were knocked down separately. The level denotes the scale at which the experiment was conducted. Block is a smaller subset of the Course data set. Course is a smaller dataset of the historical data. The Z-score was calculated using Equation 2, the result of this giving rise to the P-value. All data was generated from examining C. *elegans*.

Figure 1

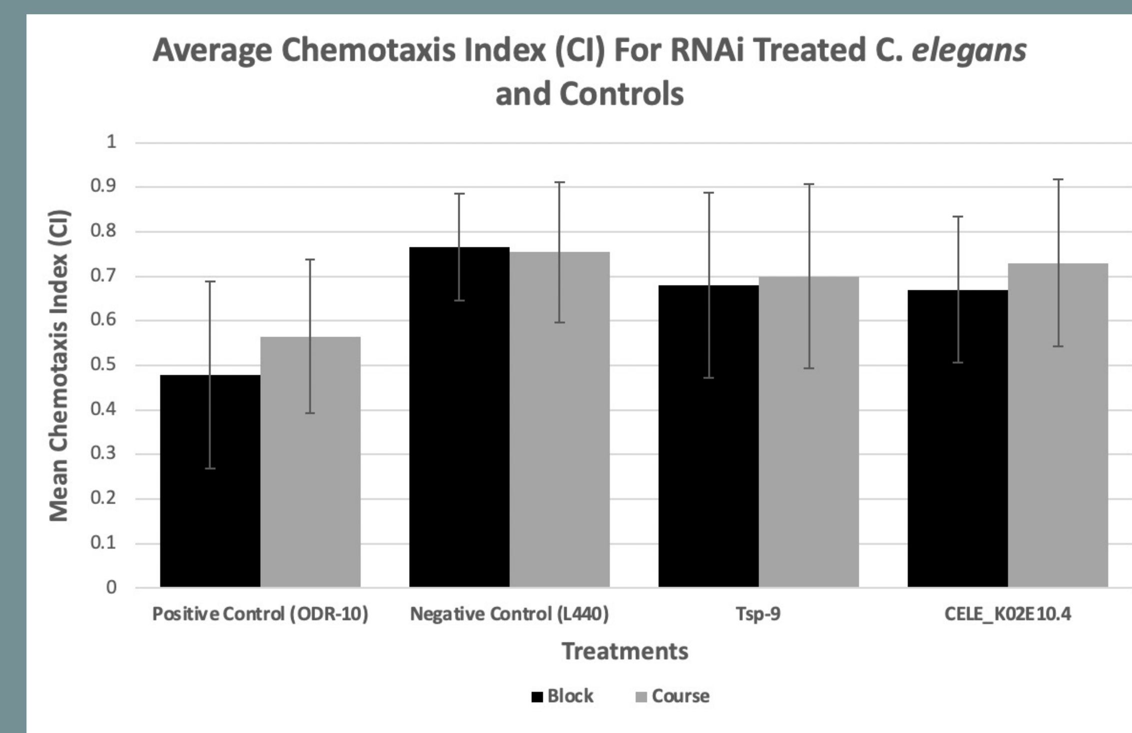

Figure 1: Average Chemotaxis Index comparing two subsets of data. ODR-10 gene was knocked down for the positive control. L440 gene was knocked down for the negative control. Tsp-9 and CELE_K02E10.4 were experimental genes that were knocked down separately. Chemotaxis values were a smaller subset for the Block reported data than the Course data. Error bars represent one standard deviation. All data was generated from examining C. *elegans*.

The data displayed in Table 1 was visualized using a clustered column graph, seen above in Figure 1. Figure 1 depicts that the largest difference in CI was between the positive (ODR-10) and negative (L440) control. This is supported by the smaller overlap in standard deviation error bars for both block- and course-collected data for ODR-10 and L440. Due to the considerable error bar overlap for Tsp-9, CELE_K02E10.4, and L440, it is suggested that there is minimal difference between the treatments, and any difference is not statistically significant. This suggestion is supported by the Z-statistic and P-value discussed previously for Tsp-9 and CELE_K02E10.4 which concluded one fails to reject the null.

Discussion & Conclusion

Utilizing the results, there is no statistically significant difference between the experimental genes, tsp-9 and CELE_K02E10.4, and the negative control, L440, in both the block and course datasets. Due to the P-value of tsp-9 being greater than 0.05 in both block- and course-collected data, the gene does not significantly affect chemotaxis toward diacetyl, and one fails to reject the null hypothesis. This negates what was inferred before experimentation, specifically that tsp-9 is involved in chemoreception and will impair chemotaxis once knocked down using RNAi. Similarly, the P-value of CELE_K02E10.4 was greater than 0.05 in both block- and course-collected data. Consequently, the gene does not significantly affect chemoattraction and one fails to reject the null hypothesis. This further contradicts predictions established before the investigation occurred.

Tsp-9, which is a protein-coding gene with human ortholog(s) of the gene involved in immunodeficiency 6, was hypothesized to be involved in chemoreception due to chemoreceptor genes and pseudogenes making up 7% of the gene complement of C. *elegans*, more than any other sequenced genome (Robertson et al., 2006). Thus, the likelihood of the gene being involved in chemoreception was thought to be high. However, tsp-9 was not found to be involved in chemoreception. Although there are no previous studies involving tsp-9 in the context of C. *elegans*, tsp-9 has human orthologs involving the immune system. This supports tsp-9 not being involved in chemoreception as it has a more distinct role in adaptive and innate immunity (The Alliance of Genome Resources, 2023). For example, tsp-9 is orthologous to the human CD81 molecule. In a previous study, it was found that CD81 regulates immune synapse, receptor clustering, and signaling, and mediates adaptive and innate immune suppression. (Vences-Catalán et al., 2017). Hence, the tsp-9 human ortholog has a distinct role in functions other than chemoreception, explaining the insignificant results obtained for the tsp-9 gene with diacetyl. As opposed to sensory reception, tsp-9 in C. *elegans* may also be involved in the immune response. Broader implications of this study include using orthologs of genes to progress the discovery of unknown genetic material through associated homologs. Future research, however, could progress analysis on the tsp-9 gene itself. Although extensive research has been conducted on orthologs of the gene, a follow-up study could provide more insight into whether the gene is involved in other processes excluding its potential in the immune response.

CELE_K02E10.4, which is a protein-coding gene predicted to be in the membrane, was hypothesized to be involved in chemoreception, also due to chemoreceptor genes and pseudogenes making up 7% of the gene complement of C. *elegans* (Robertson et al., 2006). Like tsp-9, however, CELE_K02E10.4 was not found to be involved in chemoreception. There are no previous studies on this gene, and it is largely an uncharacterized protein with no known homologs (The Alliance of Genome Resources, 2023). Thus, the result that CELE_K02E10.4 is not involved in the sensory reception of diacetyl is difficult to explain or support with past research and it is unclear whether the gene could be involved in another function as the tsp-9 gene was. However, CELE_K02E10.4's placement in the membrane could provide possible support for the result that the gene is not involved in chemoreception. Due to chemoreception genes in C. *elegans* being clustered on chromosomes, it is possible CELE_K02E10.4, while being a membrane protein as are various other chemoreceptor genes, is not part of this cluster (Robertson et al., 2006). However, this explanation is a hypothesis that would need to be tested, giving future direction to the project. A broader implication of this study includes progressing research on the CELE_K02E10.4 gene to begin the characterization of the membrane protein's biological role. An alternative route could be investigating orthologs of CELE_K02E10.4, an advantageous method in analyzing the potential function of tsp-9.

There are various origins of error in the methods and interpretations. Due to the confined space of the agar plate, the number of worms sampled per treatment was limited, contributing to a smaller, less representative sample size. Although this was corrected by using the larger dataset from the block and course, this presents inconsistency – that is, each group could have slightly varying approaches to their methodology. In the future, researchers could provide a larger propagation environment for the C. *elegans,* promoting a larger sample size and consistent methodology. Likewise, due to the confined space, propagating worms could overcrowd and compete for limited resources. If the worms did not have adequate access to bacteria, it is possible the RNAi could have no effect as the ability to propagate in the presence of the bacteria was necessary for the chemotaxis assay. In future experiments, supplying a larger environment with an abundant reserve of bacteria is necessary to avert the deficiency of resources. Additionally, the interpretation of the results in the context of previous studies presented a limitation as there was no past research on the specific genes themselves. However, continued research on these genes will establish a stronger narrative for their protein function in the future.

In conclusion, the initial hypothesis that tsp-9 and CELE_K02E10.4 are involved in chemoreception and will impair chemotaxis once knocked down using RNAi is refuted and one fails to reject the null hypothesis. The CI value alone supports this finding as Table 1 shows the mean CI for tsp-9 and CELE_K02E10.4 was 0.680 and 0.670 for block data, and 0.700 and 0.730 for course data, respectively. These values stray from a CI of 0.5 which, if seen, would indicate the genes affect chemotaxis ability due to random dispersion of worms and inability to sense diacetyl. Due to this discrepancy, however, the genes do not impact chemoattraction. This is supported by the P-values from the Z-test, all of which were greater than 0.05, indicating a lack of statistically significant difference between the experimental and the negative control genes. Overall, it is conceivable that tsp-9 is involved in immune regulation due to its human ortholog's relation to said function, whereas CELE_K02E10.4 remains an uncharacterized protein. Although neither gene was associated with chemoreception, the sparse studies on tsp-9 and CELE_K02E10.4 provide space for future exploration, utilizing the modifications outlined in the limitations.

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A special thank you to everyone who gathered and collected the data, including Dr. Alice Nguyen, head TAs, and the Intro Bio team at the University of California, Santa Barbara.